TANGERINE TOMATO CAROTENOIDs: PROCESSING, STRUCTURE, BIOAVAILABILITY AND BIOLOGICAL IMPLICATIONS OF CONSUMPTION

DISSERTATION

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ABSTRACT

Epidemiological evidence suggests people who consume diets rich in fruits and vegetables, especially diets high in tomatoes and tomato products may experience a decreased risk for developing certain cancers. Lycopene, the red pigment in tomatoes, has received the most attention for this noted decrease in cancer risk. *Tangerine* tomatoes, unique orange-colored tomatoes, accumulate lycopene in a bent configuration (*cis*-lycopene) compared to as a linear molecule (*all-trans*-lycopene) in red tomatoes. Lycopene from *tangerine* tomatoes is more similar to lycopene in the blood and tissues of people consuming lycopene rich diets. The main goal of this research is to determine if *tangerine* tomatoes are a suitable lycopene-containing food product for consideration in cancer prevention studies. We hypothesize the unique matrix of the *tangerine* tomato will play a role in its stability to processing and will result in increased carotenoid bioavailability in humans and bioefficacy in chemoprevention in animals. A comparison of red and *tangerine* tomatoes (two very similar foods with very different forms of lycopene) offers an opportunity to compare properties of *all-trans*-lycopene and *cis*-lycopene isomers.

The objective of these studies are to: determine the physical storage form of lycopene in *tangerine* tomatoes, investigate the stability of lycopene from *tangerine* tomatoes to thermal processing, determine if lycopene from *tangerine* tomatoes is more bioavailable.
in humans compared to red tomatoes and determine if consuming tomatoes can decrease tumor incidence in mice exposed to UV radiation.

Light and transmission microscopy demonstrated that lycopene is stored in non-crystalline bodies in *tangerine* tomatoes (a potentially more bioavailable form), compared to as crystals in red tomatoes. Lycopene from *tangerine* tomatoes is more sensitive to thermal processing compared to red tomatoes. Lycopene from *tangerine* tomatoes is, on average 8.5 times more bioavailable compared to from red tomatoes (*P* < 0.0001). Our results additionally suggest male mice consuming tomatoes and exposed to UVB radiation develop less non-melanoma skin tumors than mice on control diets.

These findings confirm, and provide rationale for the enhanced bioavailability of *cis*-lycopene from *tangerine* tomatoes and support further research to explore *tangerine* tomatoes to deliver lycopene in future studies targeted toward diet and human cancer prevention.
Dedicated to my family.

And, to anyone who reads this document. I hope it’s informative and helpful.
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1.1 Diet, nutrition and chronic disease

There is an inextricable link between one’s diet and lifestyle habits, and risk for chronic diseases. In a landmark paper from the early 1980s, Doll and Peto estimated that up to 30% of cancer deaths could be attributed to nutritional factors, an equal number deaths as can be attributed to tobacco use (1). There is consistent evidence in the literature that increased consumption of fruits and vegetables is protective against cancers of the stomach, esophagus, lung, oral cavity/pharynx, endometrium, pancreas and colon (2). Raw vegetables, allium vegetables (e.g. onions, garlic, leeks), carrots, green vegetables, cruciferous vegetables (e.g. broccoli, kale, Brussels sprouts) and tomatoes are most associated with this decreased risk (2). Similar data exists for heart disease, suggesting a link between increased fruit and vegetable consumption and a decreased risk of cardiovascular disease (3,4).

1.1.1 Epidemiology of carotenoids

A relationship between carotenoids and cancer risk was first observed in 1981 (5). Peto et al. suggested that β-carotene may be able to reduce human cancer rates, and may act in
some way beyond its provitamin A activity. Since then, many epidemiological investigations have been launched correlating diets high in carotenoids or elevated blood plasma carotenoid levels with decreased risk for disease. There have been correlations between carotenoid levels and breast cancer (6), colorectal cancer (7,8), prostate cancer (9) and lung cancer (10), just to mention a few.

1.1.2 Epidemiology of tomato products
Tomatoes are the most commonly consumed canned vegetable in the American diet. Tomatoes account for 22% of the vegetable consumption in the United States, with over 75% of the tomatoes being consumed in a processed form (i.e. canned, salsa, juice, ketchup) (11). Consumption of a tomato-rich diet has been associated with decreased risk for a variety of chronic diseases, including heart disease and cancer, specifically prostate cancer (12,13). Inflammation has been implicated as playing a critical role in the development of many of these diseases. Tomatoes contain lycopene, a carotenoid pigment that imparts their red color. Lycopene is often identified as being responsible for the noted decreased risk of disease in those consuming tomato products (14). However, we propose that it is the combination of several components of tomatoes that result in the observed protection, suggesting the need for a whole food research approach rather than a reductionist approach. Notably, tomatoes also contain other carotenoids upstream in the synthesis of lycopene (including phytoene and phytofluene) which may also confer health benefits (15).
1.2 Carotenoid chemistry

Carotenoids are a class of pigments ranging from yellow to red in color and are found ubiquitously in plants (both edible and non-edible) and photosynthetic microorganisms. They are the most widely distributed pigments in nature (16). Carotenoids are responsible for the red color in tomatoes, orange in sweet potatoes, and yellow in squashes. They are 40 carbon highly unsaturated hydrocarbons derived from isoprene units and composed either entirely of carbon and hydrogen (carotenes) or carbon, hydrogen, and oxygen (xanthophylls). There are over 750 known carotenoids to date (17). One function of carotenoids in plants is to help aid in photosynthesis, mainly by absorbing light and protecting against photosensitization (18). Additionally, the carotenoid biosynthetic pathway also produces abscisic acid, a critical plant hormone involved in plant growth and response to environmental stress (19). Some carotenoids have provitamin A activity (e.g. β-carotene, α-carotene, and β-cryptoxanthin; those carotenoids with an unsubstituted β-ionone ring) while most do not (e.g. lycopene, lutein, zeaxanthin). Additionally, consumption of carotenoid rich foods (including those containing carotenoids that cannot be converted to vitamin A) has been associated with a decreased risk of disease (20) including cancer (5) and cardiovascular disease (21).

Carotenoids can act as antioxidants by reacting with free radicals, including singlet oxygen and peroxyl radicals (22,23). Since carotenoids are highly unsaturated pigments, many possible cis/trans isomers are possible, both of which have biological implications, depending on the carotenoid of interest.
1.2.1 Carotenoid cis/trans isomers

Carotenoids, since they are long unsaturated polyene chains, can exist in a number of cis/trans configurations. Carotenoids usually contain 9 to 11 conjugated double bonds, but may contain as few as 3 and as many as 15 (24). Lycopene contains 11 conjugated (and 2 unconjugated) double bonds and can theoretically form 1056 geometrical (cis/trans) isomers. However, steric hindrance favors the formation of those isomers that exist in the lowest energy state, and the true number of geometrical isomers is likely significantly less (25). In raw, red tomatoes, approximately 95% of the lycopene present is in the all-trans form (26). In contrast, cis-isomers account for 58-73% of total lycopene in serum, and a surprisingly high 79-88% of total lycopene in benign or malignant prostate tissue (27). It is generally accepted that all-trans-lycopene is converted to cis isomers in vivo, and/or cis isomers are more bioavailable and thus preferentially absorbed compared to all-trans (28). The cis isomers tend to be more polar, less likely to crystallize and more oil/hydrocarbon soluble compared to the all-trans forms, (29) while being preferentially micellarized (30) and taken up by intestinal cells (31).

*Tangerine* tomatoes are one of the few fruits or vegetables that naturally accumulate high concentrations of cis-lycopene. Commonly occurring isomers of lycopene are shown in Figure 1.1. In contrast to red tomatoes, *tangerine* tomatoes are a unique cis-lycopene rich type of tomato developed through conventional breeding techniques. These tomatoes have the recessive mutation *tangerine* (32) and lack a functional form of the enzyme
carotenoid isomerase (CRTISO), which converts poly-cis- into all-trans-lycopene and is necessary for the biosynthesis of downstream cyclized carotenoids (33). As a result, tangerine tomatoes accumulate several cis-lycopenes, with tetra-cis-lycopene ((7Z, 9Z, 7′Z, 9′Z)-lycopene, also called prolycopene) predominating at the expense of the all-trans form. Tetra-cis-lycopene absorbs light maximally approximately 35 nm below all-trans-lycopene, resulting in tomatoes with an orange color as shown in Figure 1 (34). Most processed foods containing tomato have concentrations of cis isomers less than 10% (35) although severe food processing conditions can increase the percentage of cis-lycopenes, shown to increase bioavailability (36). Stemming from this result, others have suggested tetra-cis-lycopene may be more bioavailable than all-trans (37).

Conversely, there is evidence in mice and humans that all-trans-β-carotene is preferentially absorbed over cis forms, and cis forms in a bolus dose can be converted to the all-trans form in vivo (38–40). 9-Cis and 13-cis-β-carotene are converted to vitamin A with 38 and 53% efficiency, respectively, compared to all-trans-β-carotene (41). Regardless, the isomeric form of carotenoids plays an important role in their biological function.
Figure 1.1 - Structures of all-trans-lycopene and commonly occurring cis-lycopene isomers.
1.3 Biosynthesis of carotenoids in plants

Carotenoids are important secondary plant metabolites produced ubiquitously in plants. They were first described in the literature in the early 19th century as sensitive, lipophilic pigments providing color to paprika, saffron, annatto, carrots and autumn leaves (17). Isoprenoids are synthesized via two different pathways: the mevalonic acid (MVA) pathway and the MVA-independent pathway, also called the MEP (for 2-C-methyl-D-erythritol 4-phosphate) pathway (42). Originally it was thought that carotenoids were synthesized from MVA, but it has since been discovered that isoprenoids in plastids (including carotenoids, phylloquinones, tocopherols, chlorophylls and gibberellins) are made via an MVA-independent pathway (43,44). Figure 1.2 shows a schematic summarizing the biosynthesis of carotenoids. In the MVA pathway, three acetyl-CoA molecules form a 3-hydroxy-3-methyl-glutaryl coenzyme A (HMG-CoA) (42). HMG-CoA can be reduced to MVA via HMG-CoA reductase (HMGR) (42). Experiments conducted in transgenic tobacco plants have shown that sterols, but not carotenoids are limited by the activity of HMGR, indicating they may be synthesized another way (45). Additionally, mevinolin, which is an inhibitor of HMGR, inhibits synthesis of sterols (which are synthesized via the MVA pathway) but does not affect the synthesis of carotenoids or chlorophylls, giving more evidence for two separate pathways (45). Cytosolic and mitochondrial isoprenoids are made via the MVA pathway (43).
Figure 1.2 - Simplified representation of the biosynthesis of carotenoids in higher plants, adapted from Fraser and Bramley (43) and Khudairi (46).
The first step in the synthesis of carotenoids (and other isoprenoids) is the formation of isopentenyl diphosphate (IPP) (43). Pyruvate and D-glyceraldehyde-3-phosphate are condensed head to head to yield 1-deoxy-D-xylulose 5-phosphate (DXP) (43). DXP can be converted to MEP by DXP reductoisomerase with NADPH and Mn$^{2+}$ as cofactors (43). MEP is converted to dimethylallyl diphosphate (DMAPP) and IPP by 2-C-methyl-D-erythritol 4-phosphate cytidyl transferase (MCT), 2-C-methyl-D-erythritol 2,4-cyclodiphosphate (MCS) and 1-hydroxyl-2-methyl-2-(E)-butenyl 4-phosphate synthase (HDS) (43). Next, IPP is converted to geranyl pyrophosphate (GPP) and then to geranylgeranyl pyrophosphate (GGPP) to phytoene (43). IPP, a five carbon compound can be isomerized to DMAPP by IPP/DMAPP isomerase, and the two are condensed head to tail to form GPP with the help of geranyl diphosphate synthase (43). Another IPP can be added to GPP to form the 15 carbon farnesyl pyrophosphate (FPP), to which one final IPP can be added to yield GGPP, via geranylgeranyl diphosphate synthase (43). Two GGPP molecules are linked head to head to form a pre-phytoene pyrophosphate (PPPP) by phytoene synthase (PSY) (43). Phytoene results from the elimination of the diphosphate group and the abstraction of a specific proton from the PPPP molecule (43). Phytoene is the precursor for all other carotenoids (both straight chain and cyclized) (43).

Phytoene, a colorless carotenoid absorbing maximally at 286 nm, often exists in the 15-cis form in plants (43). The conversion to GGPP to phytoene is thought to be the rate limiting step in carotenoid synthesis in many plants (47). In the non-carotenogenic
*Escherichia coli* with an inserted PSY cDNA, only one enzyme is required to condense GGPP to phytoene (48). Genes that code for PSY have been found in many different plants, including carrot, corn, melon, pepper, marigold and *Arabidopsis* (43). PSY 1 (from chromoplasts) and PSY 2 (from chloroplasts) from tomatoes and peppers are well characterized (49,50). Four desaturations allow the conversion of phytoene to phytofluene, ζ-carotene, neurosporene and finally prolycopene/lycopene, the first two desaturations governed by phytoene desaturase (PDS) and the second two by ζ-carotene desaturase (ZDS) (43). Since most phytoene is in the 15-*cis* form, it is accepted that some *cis-trans* isomerization must occur *in vivo* (33). CRT I performs all four desaturations in bacteria and fungi (43).

The enzyme carotenoid isomerase (CRTISO) is responsible for converting prolycopene (7,9,7',9'-*cis*-lycopene) into all-*trans*-lycopene (33). The *tangerine* gene in tomatoes has been incredibly useful in studying how carotenoids isomerize *in vivo*. *Tangerine* tomatoes have a deletion in the 348 bp promoter for CRTISO, accumulating tetra-*cis*-lycopene at the expense of all-*trans* (33). This alteration in the chromophore of lycopene imparts the characteristic orange color of *tangerine*-type tomatoes (25). CRTISO is a redox-type enzyme and is highly homologous to CRT I in bacteria and another gene in *Arabidopsis* and cyanobacteria that plays a role in carotenoid isomerization (33). When comparing wild-type (red) tomatoes to *tangerine* tomatoes, at 7 days after the breaker stage, over 75% of tomato carotenoids are all-*trans* while less than 15% are lycopene precursors (phytoene, phytofluene, ζ-carotene and neurosporene). However, in *tangerine*
tomatoes, most lycopene is in the *cis* form, with less than 2% of lycopene as all-*trans*, and tetra-*cis*-lycopene and lycopene precursors predominating. In wild-type flowers of tomato plants, xanthophylls predominate, with neoxanthin, violaxanthin and lutein composing over 95% of the flower carotenoids, however in *tangerine* flowers, tetra-*cis*-lycopene and precursors accumulate and xanthophylls range from 10-50% of total carotenoids. It is hypothesized that this is because lycopene cyclases cannot operate on tetra-*cis*-lycopene because of steric hindrance. The bacterial lycopene cyclase CRTY (which converts all-*trans*-lycopene to β-carotene) cannot cyclize tetra-*cis*-lycopene (33). Additionally, it is hypothesized that light can isomerize tetra-*cis*-lycopene into all-*trans* (51). Figure 1.2 shows ripe wild-type (red) and *tangerine* tomatoes.
Figure 1.3 - Wild-type (red) tomatoes (left) and *tangerine*-type tomatoes (right). Photos by Jessica Cooperstone
1.4 Biological functions of carotenoids

Carotenoids are integral pigments, critical to the survival and growth of photosynthetic tissues. They function in light harvesting complexes, act as accessory pigments to light harvesting, allow for photoprotection, dissipation of excess energy and scavenging of singlet oxygen and reactive oxygen species to prevent plant damage, are necessary for the downstream production of abscisic acid (52). Additionally, they are important in providing the yellow, orange and red colors to avian plumage and play a role in mate selection and signal to other birds a state of immunocompetence (53). It has also been suggested that increased deposition of carotenoids in skin may also make a person more attractive (54).

The function of carotenoids in humans may or may not be related to their antioxidant function. Lycopene has been shown to be the most effective singlet oxygen quencher \textit{in vitro} (55). Lycopene, carotenoids, and carotenoids in mixtures can also inhibit the formation of oxidation byproducts, like thiobarbituric acid-reactive substances (56). However, there is some evidence that purified carotenoids given at supraphysiologic doses in people who already have high levels of oxidation (e.g. smokers) can be detrimental (57,58). \textit{In vivo} studies have shown that a tomato rich diet can decrease LDL sensitivity to oxidative damage (59–61). In addition, serum thiobarbituric acid-reactive substances were decreased in serum of those supplemented with a lycopene rich tomato product for one week (62).
Carotenoids are also thought to induce phase II enzymes, potentially aiding the body in protection against chronic diseases via detoxification mechanisms (63). Carotenoids (especially lycopene) have been shown to activate antioxidant response elements (AREs) and this activation was not solely due to the carotenoid’s activity as an antioxidant (64). A lycopene metabolite (apo-10’-lycopenoic acid) has been shown to activate nuclear factor E2-related factor (Nrf2) which is associated with the induction of a number of phase II detoxifying enzymes (65).

Carotenoids and lycopene may provide health benefits in their ability to modulate immune function. A recent study has shown that adding tomatoes to a high fat meal can modulate post-prandial induction of an inflammatory cascade (66). A study of 106 overweight or obese women showed that serum levels of both IL-8 and TNF-α post-intervention and IL-6 was decreased in the obese cohort (67).

1.5 Lycopene and thermal processing

Over 75% of tomatoes in the United States are consumed in the form of processed tomato products (68). The effects of thermal processing on carotenoid profiles in red tomatoes have been extensively studied. Lycopene from tomato products seems to be relatively stable to moderate heat processing, especially in the absence of fat (35,69). Extensive heat processing can cause degradation (68). It has been shown that lycopene is more stable in a tomato matrix, compared to isolated, purified or in solvents (50). The effects of fat on lycopene isomerization from tomatoes have yielded mixed results. The addition
of 5% or 15% olive oil has been shown to not affect lycopene isomer profile (35) while tomato juice heated in an oven at 180 °C with 10% safflower showed significant isomerization (71). Schierle et al. demonstrated that tomato dissolved in water underwent less isomerization compared to tomato dissolved in oil olive, when subjected to 3 hours of heating at 75 °C (72).

1.6 Carotenoid absorption

Carotenoids must first be released from the food matrix before they can be incorporated into mixed micelles and absorbed by the body. A schematic of carotenoid absorption can be found in Figure 1.4. Micelles contain bile salts, cholesterol, and fatty acids from the meal, and the amphipathic nature of the micelle structure helps to keep the lipophilic nutrients soluble in the aqueous digesta (73). The micelles approach the unstirred water layer of the apical side of the intestinal cells (enterocytes), and move across the apical membrane (73). Conventionally, lycopene and other carotenoids were thought to be absorbed via passive diffusion although recent work has shown that this absorption is also facilitated by cholesterol membrane transporters, including scavenger receptor class B type I (SR-B1) (74,75). Chylomicrons are then transported across the basolateral membrane and make their way into the lymphatic system, which eventually releases chylomicrons into the blood. Bioavailability of a carotenoid is defined as the amount of the carotenoid which is absorbed, metabolized, packaged into chylomicrons and released into circulation after consumption of a carotenoid-containing meal. As a result, bioavailability of carotenoids can be monitored by measuring levels in the chylomicron
Figure 1.4 - Schematic of carotenoid absorption, adapted from Yokenura et al. (76).
fraction (also called the triglyceride rich lipoprotein fraction, or TRL fraction) of plasma. Bioavailability of carotenoids can be affected by a number of factors, including food processing and dietary composition (77). After carotenoids are assembled into chylomicrons by the Golgi apparatus in the enterocyte for secretion into the lymph, they are degraded by lipoprotein lipase and become chylomicron remnants (78). At this point, carotenoids can be taken up by extrahepatic tissues or delivered to the liver where they can be stored or released on very low density lipoproteins (VLDLs) (78). Next, similarly to other lipids, VLDLs are subject to removal of lipid to form low density lipoproteins (LDLs) where carotenes primarily circulate in the blood, and can be also released and taken up by tissue (78). The precise process of the delivery of carotenoids from the blood to extrahepatic tissues, or release from the extrahepatic tissues back into circulation, is not well understood.

1.7 Factors affecting lycopene bioavailability

De Pee and West have proposed the acronym SLAMENHI to describe the different factors affecting bioavailability and bioconversion of carotenoids, which was further reviewed by Castenmiller and West (29). SLAMENHI refers to S: species of carotenoids, L: molecular linkage, A: amount of carotenoid, M: food matrix, E: effectors of absorption/conversion, N: nutrient status, G: genetic factors, H: host-related factors, I: interactions.
In general, all-\textit{trans}-beta-carotene is more bioavailable compared to \textit{cis} forms (39,40,79), while \textit{cis}-lycopene is more bioavailable than all-\textit{trans}-lycopene (36,80). Also, it is thought that xanthophylls are more bioavailable than hydrocarbon carotenotes because of their increased polarity (81). A few studies have shown that xanthophyll esters are equally (82), or more bioavailable than free xanthophylls (83), although this does not directly apply to lycopene since it does not form esters. The fractional dose of lycopene absorbed decreases as the dose of lycopene increases (84) and there is some evidence of competition for absorption (85–87), but this observation is not consistent among all studies and all carotenoids.

The matrix of the carotenoid plays a large factor in absorption. \(\beta\)-carotene is approximately more bioavailable from fruit (papaya, mango and pumpkin) than from vegetables (green leafy vegetables, carrots) (88). Lycopene has shown to be better absorbed from processed tomato products, compared to those that are raw (80,89). This suggests that there is something about the physical form of carotenoid that can act as a barrier to absorption. Fat has also been shown to play a critical role in aiding absorption, likely for its ability to solubilize carotenoids (90–92). Additionally, fatty acid profile is important (93,94) and the presence of synthetic lipids like olestra, also decrease carotenoid absorption (95). Fiber has been shown to decrease carotenoid absorption (96).

The nutrient status of the host is important for provitamin A absorption and conversion, but less relevant to lycopene absorption. There are studies that show that SNPs can
influence carotenoid absorption (and in the case of provitamin A carotenoids, conversion) (97–99). Most studies in the literature report higher free-living serum carotenoid levels in women [43, 44], likely a function of higher consumption of carotenoids in the diet. Additionally, it has been shown that chronic supplementation of β-carotene over one year leads to a relatively higher increase in plasma β-carotene in women (102). It has been suggested that for retinol, women have a higher transfer coefficient from the plasma to the extravascular pool, and this faster clearance is responsible for an apparent lower appearance of $^{13}$C labeled retinyl palmitate derived from $^{13}$C labeled β-carotene or retinyl acetate (103).

### 1.8 Physical storage forms of carotenoids

In order for carotenoids to exert their potential beneficial health effects in animals or humans, they must be in a form that can be absorbed. There is considerable research investigating differences in bioavailability within carotenoid species (29). But, the physical state in which carotenoids are stored in a plant can greatly influence carotenoid bioavailability in vivo. Physical state of carotenoids in plants varies widely, even within the same species. Carotenoids can occur in several forms in fresh plant foods, including in carotenoid-protein complexes in chloroplasts, crystalline form inside chromoplasts or in lipid dissolved droplets called plastoglobuli (104). Crystalline structures are difficult to solubilize, while carotenoids associated with lipids may be more bioaccessible. These lipid dissolved carotenoids could be more easily removed from the food matrix and thus theoretically, more available for absorption by the enterocyte [22, 23]. Carotenoid
physical storage form in chromoplasts, has been blamed for the relatively low bioavailability of raw, green leafy vegetables like spinach; a function of carotenoids being tightly bound to protein complexes within plant cells [24, 25]. Physical state of carotenoids in plants varies widely, within the same plant species and even within different parts of the same plant (108), imparting large heterogeneity in carotenoid storage within plants (109). Lycopene is red tomatoes is stored as crystals (110) and it has been suggested the lycopene in tangerine tomatoes is stored in globular chromoplasts (108).

In common red tomatoes, lycopene is found in large crystalline aggregates of up to 15 μm length within chromoplasts, the cellular organelle where carotenoids are biosynthesized and deposited (105). Carotenoid liberation and solubilization from such crystals was hypothesized to be significantly lower when compared to lycopene from smaller aggregates [23]. This suggests that chromoplast morphology may play a role in post-prandial bioavailability of carotenoids. Non-crystalline deposition of lycopene is rarely found in natural plant foods since all-trans-lycopene easily crystallizes. Therefore, most common lycopene containing fruits (red tomatoes, watermelon, and red-fleshed papaya) have crystalline lycopene aggregates (105,108). Often, in order to have lipid-dissolved carotenoids a high concentration of fat is required, as previously reported for carotenoids in peach palm (Bactris gasipaes Kunth) fruits (111). Although tangerine tomato contains only minor amounts of fat, tetra-cis- and other cis-isomers are expected to be deposited in a non-crystalline form. A lipid-dissolved deposition state within small
lipid globules (plastoglobules) of *tangerine* chromoplasts has been suggested (108), although simultaneous investigations of the carotenoid profile and the chromoplast ultrastructure has not been conducted.

1.9 Carotenoids and skin

It is thought that carotenoids may play a role in protection of the skin against oxidative damage. The sun emits ultraviolet (UV) radiation from approximately 100-400nm, which can be further divided into UVA (315-400 nm), UVB (250-315 nm and UVC (~100-280 nm) (112). UVB light appears to be much more effective in producing cancer in animals compared to UVA light, while most UVC light is filtered out by the atmosphere (113). Damage from UV radiation can cause reactive oxygen species (ROS) when light of a suitable wavelength interacts with a chromophore. Photooxidative damage can affect lipids proteins and DNA and is involved in skin aging, erythema, photodermatoses and skin cancer (114). An individual’s sensitivity to UV radiation can be assessed by determining a minimal erythemal dose (MED), or the dose needed to cause reddening of the skin 24 hours after exposure to UVB radiation (115). MED levels can differ wildly between individuals and are related to skin type, categorized using the Fitzpatrick scale from type I to type IV. Skin type I denotes people with white or freckled skin, light blue or green eyes, light blonde or red hair with a high sensitivity to sunburns while skin type IV denotes people with black skin, dark eyes and black hair, almost never suffering sunburns. Melanin levels play a large role in an individual’s susceptibility to UV-induced damage, as melanin scatter and absorb UV light (116).
It is hypothesized that carotenoids act as photoprotectants (117), free radical quenchers (55) and antioxidants (118), all of which may play a role in protecting the skin against UV-induced damage. Since carotenoids are accessory pigments in plants and play a role in protecting plants against damage from excess light, it is logical to think that carotenoids may impart some photoprotective properties in animals. Studies in mice that are both hairless and immunocompetent (Skh-1) have shown that β-carotene and canthaxanthin can be protective against UVB induced damage (119–122). Some evidence from human clinical trials suggest that lycopene and tomato paste can decrease erythema from UV light (123). Sies and Stahl conducted a number of experiments that suggest tomato-derived carotenoids can protect against UV-induced erythema. Sies and Stahl have conducted five studies (tomato paste, carrot juice, lycopene supplement, lycopene drink and synthetic lycopene) in type II skin type individuals using various carotenoid diets for 10-12 weeks. In the tomato paste study, subjects consumed 40 g of tomato paste with 10 g olive oil providing 16mg of lycopene per day (123). In the carrot juice study, subjects consumed 400mL of a unique carrot juice providing 10 mg lycopene and 5.1 mg β-carotene (114). In the lycopene supplement study, subjects consumed a soft gel capsule with tomato extract providing 9.8 mg lycopene and 0.4 mg β-carotene per day (124). In the lycopene drink group, subjects consumed a lycopene drink derived from tomato extract, containing 8.2 mg of lycopene and 0.4 mg β-carotene per day (124). The synthetic lycopene study had subjects consume 10.2 mg of synthetic lycopene per day (124). All studies led to increases in serum carotenoids and a less substantial
increase in skin carotenoids as determined by HPLC and Raman spectroscopy respectively. After 10 or 12 weeks of supplementation, all groups except the synthetic lycopene had a significant decrease in Δa (a quantitative change in the redness of skin) as compared to baseline. This suggests that other carotenoids in tomato products besides lycopene (including phytoene and phytofluene) may play a role in protection against UV-induced erythema.

1.10 Carotenoid analysis by HPLC

Carotenoids have been extensively studied plant pigments for almost 200 years and have helped to facilitate the development of the field of chromatography (17). The first methods for separating carotenoids and chlorophylls using open column chromatography were developed as early as 1906 (125). High performance liquid chromatography (HPLC) analysis for foods has been extensively reviewed (126).

For the past few decades, HPLC has been the preferred method of separating, identifying, and quantifying carotenoids in foods and biological samples. The photodiode array (PDA) is the most commonly used detector for HPLC carotenoid analysis, although other detectors, like electrochemical detectors (ECD), fluorescence, mass spectrometers (MS), and nuclear magnetic resonance (NMR) can be used. Reviews on PDA detectors in relation to carotenoid analysis can be found elsewhere (127). Advantages of HPLC include efficiency, short run times, and sensitivity (128). Reversed-phase HPLC is by far most common, although some normal phase methods exist. Normal phase methods have
been developed for separation of mixtures of xanthophylls or carotenoid ketones, like astaxathin (129), with little affinity for a C18 or C30 “carotenoid” column. Some apocarotenoids like bixin and norbixin are much shorter in chain length and more polar than a typical xanthophyll and require alterative solvent systems (130). C18 columns are often sufficient for separating different carotenoids although C30 columns tend to yield better separations, especially of very similar compounds (eg. lutein and zeaxanthin) (131). Additional methods for separation of carotenoids/carotenoid isomers are discussed in the cis/trans isomer portion of this chapter. These methods are sufficient to separate carotenoid species, but often require longer run times. Ultra high performance liquid chromatography (UHPLC) has been used to monitor at carotenoids, but mostly in conjunction with other fat soluble vitamins (132,133). UHPLC methods for quantification of a wide range of carotenoids are uncommon. Column temperature should be controlled and maintained above 20°C to promote consistent separation, and prevent carotenoid crystallization out of solution (134,135).

Most of the early published work on the separation of carotenoids by HPLC employs C18 stationary phases. It has been shown that polymeric surface modified C18 columns are more selective towards isomers compared to monomerically bound columns (136). In the 1980’s and 1990’s, a 30 carbon bound stationary phase (C30 column) was developed and coined the “carotenoid column,” because of its shape selectivity and superior ability to separate not only different carotenoid species, but also isomers (136). Elution order of lycopene varies greatly between C18 and C30 columns. On a C18 column lycopene
elutes before α-carotene and β-carotene. In contrast, on a C30 column lycopene is retained and elutes last, allowing efficient separation of isomers (137).

A C30 column can be used to distinguish between all-trans-lutein and all-trans-zeaxanthin and their cis isomers (138), β-carotene and β-carotene cis isomers (139), and lycopene and cis-lycopene isomers (140). C30 columns can allow separation of isomers induced by heat processing (and induced in vivo. Extensive reviews on the development of C30 stationary phases for carotenoid analysis have been written by Lane Sander (136,137).

Most C30 methods use some combination of methyl-tert butyl ether (MTBE), methanol, and a small amount of water and employ a gradient for optimum separation of different carotenoid species and their isomers (141–143). Some C30 methods can also separate carotenoids/carotenoid isomers while simultaneously separating tocopherols and chlorophylls (144). Others use a combination of acetonitrile, methanol, isopropyl alcohol, ethyl acetate, dichloromethane or THF, and combinations thereof (131,145–148). Ammonium acetate (0.05 M) and/or triethylamine (0.05%) have been shown to improve on-column recovery and buffer acidic uncapped silanol groups of the column backbone (131,148,149). Column temperature can also affect chromatographic separations, generally 23 °C ± 1 °C is sufficient to separate different carotenoid species but 30 °C has been found to be ideal for separating isomers (135). BHT is sometimes added as an antioxidant. A “cis peak” (denoted A_B) is often visible in the spectra of a cis
carotenoid as an additional peak 142nm below the wavelength of maximum absorbance ($A_{11}$) (150,151). The intensity of the cis peak increases when it is located toward the center of the chromophore. Additionally, the appearance of a double-cis peak may indicate an aliphatic or monocyclic chromophore (150,151). Generally, cis isomers tend to absorb maximally at wavelengths approximately 2-6 nm below that of the all-trans carotenoid and have a reduction in fine structure overlaid (152). Figure 1.5 shows overlaid spectra of all-trans-lycopene and two lycopene cis isomers. In order to unequivocally identify the structure of a specific carotenoid geometrical isomer (eg. 9-cis-lycopene) to a peak in a chromatogram, nuclear magnetic resonance (NMR) is used (153).
Figure 1.5 - Overlaid spectra of all-trans-lycopene, prolycopene (also called tetra-cis-lycopene) and 13-cis-lycopene (154).
1.11 Specific aims

*The main goal of this research is to determine if tangerine tomatoes are suitable lycopene-containing food product to be considered in cancer prevention studies.* We hypothesize that the unique matrix of the *tangerine* tomato will play a role in its stability to processing and will result in increased carotenoid bioavailability in humans and bioefficacy in chemoprevention in animals.

This will be achieved by investigating: 1) the physical storage form of carotenoids in *tangerine* tomatoes, 2) how stable carotenoids are to thermal processing in *tangerine* tomato juice and sauce, 3) the bioavailability of carotenoids in *tangerine* tomato juice to the bioavailability of carotenoids in a conventional red tomato juice in humans and 4) whether dietary consumption of *tangerine* and red tomatoes can modulate tumor promotion and progression in a skin cancer model of mice.

Understanding the physical storage form of carotenoids in *tangerine* and conventional red tomatoes, may provide insight into differences in bioavailability. Investigating stability of *tangerine* tomato carotenoids to thermal processing will allow development of tomato products with optimal carotenoid profiles. A human clinical trial comparing absorption of dose-matched *tangerine* and red tomato juices will determine if differences in bioavailability of carotenoids between the two juices exist. A mouse study investigating chronic UVB exposure in mice consuming control, *tangerine* or red tomato diets will
give insight as to the bioefficacy of *tangerine* and red tomato carotenoids to reduce skin cancer *in vivo*.

**Aim 1**: Characterize the ultrastructure of carotenoid containing bodies of red and *tangerine* tomatoes.

We will use light and transmission electron microscopy (TEM) to determine physical storage forms of carotenoid in red and *tangerine* tomatoes. Observed crystal structure and storage forms will be correlated to carotenoid profile and content in these tomatoes.

Our hypothesis for aim 1 is that tomatoes high in all-\textit{trans}-carotenoids will accumulate these carotenoids in crystalline forms, while tomatoes that accumulate \textit{cis}-carotenoids will accumulate these carotenoids in plastoglobules, lipid dissolved droplets or otherwise in a noncrystalline fashion.

**Aim 2**: To investigate the effects that thermal processing and fat levels (both separately and combined) have on isomerization and content of lycopene and lycopene precursors (phytoene, phytofluene, ζ-carotene and neurosporene) in a *tangerine* tomato products.

We will conduct a series of experiments using a 4x4 (juice) and 5x5 (sauce) Latin Square factorial design to test the effects on increasing time of thermal processing and increasing
(olive oil) fat concentration (as well as the interaction between the two) on carotenoid content.

Our hypothesis for aim 1 is that tetra-cis-lycopene will decrease with increased processing time and all-trans-lycopene and other-cis-lycopene will increase with processing.

Aim 3: To determine if a processed *tangerine* tomato juice has enhanced bioavailability of carotenoids and flavonoids compared to a commercially available processed red tomato product in humans and if tomato consumption, in the short-term, can deliver bioactive compounds that will impact markers of inflammation in humans.

We will conduct a randomized, crossover designed clinical trial in humans. Each subject will consume both a red and *tangerine* tomato juice and the appearance of carotenoid and flavonoids in blood will be monitored over time. We hypothesize that carotenoids will be more bioavailable from *tangerine* tomato juice compared to red tomato juice. Additionally we hypothesize that flavonoids will be bioavailable from both *tangerine* and red tomato juices. Additionally, we hypothesize that feeding of either type of tomato will impact markers of inflammation (including IL-6) in humans.
**Aim 4:** To determine whether the dietary consumption of *tangerine* tomatoes or red tomatoes will differentially reduce the UVB-induced tumor promotion and progression after chronic UVB exposure in male and female Skh-1 murine skin.

We will use 4 week-old male and female Skh-1 hairless mice, which will be fed one of the following 3 AIN-93G based diets for 10 weeks; 1) control diet 2) 10% *tangerine* tomato 3) 10% red tomato. At the beginning of week 11, mice on their respective diets will be exposed to a UVB treatment three times weekly on non-consecutive days for an additional 25 weeks and sacrificed at 48h following the last UVB exposure. This treatment protocol induces papilloma growth and ultimately results in squamous cell carcinoma development at approximately 25 weeks. The effect of tomato supplementation on tumor parameters (number, burden and grade) and carotenoids in mouse serum and tissue will be determined.

Our hypothesis for aim 4 is that consumption of tomatoes containing lycopene, phytoene, and phytofluene will reduce inflammation and skin damage, and tumor development caused by UV radiation as compared to no tomato consumption. Additionally, we hypothesize that there will be a dose-response relationship such that diets containing freeze dried *tangerine* tomato powder containing high levels of phytoene and phytofluene will confer a greater level of protection against UV damage than red tomato products containing an intermediate level of phytoene and phytofluene compared to control feed.
CHAPTER 2 - THE EFFECTS OF PROCESSING ON DEGRADATION AND ISOMERIZATION OF CAROTENOIDS IN \textit{TANGERINE} TOMATO PRODUCTS

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2.0 Abstract

Increased tomato consumption has been associated with a decreased risk of chronic diseases. Almost all lycopene in red tomato products is present in the all-\textit{trans} configuration while blood and tissues contain primarily \textit{cis}-lycopene. The \textit{tangerine} tomato is a unique variety that accumulates \textit{cis}-lycopene instead of the conventional all-\textit{trans}. Our objective was to understand how thermal processing and level of fat affects carotenoid isomerization and degradation in \textit{tangerine} tomato products. We conducted factorial designed experiments in duplicate producing \textit{tangerine} tomato juice containing 0, 1, 2 or 3\% olive oil heated for 0, 30, 60 or 120 min after come up to 88 °C, and heated \textit{tangerine} tomato sauce with 0, 1, 5, 15 or 30\% olive oil heated for 0, 30, 60, 120 or 180 min after come up to 100 °C. \textit{Tangerine} tomato juices and sauces were then extracted for carotenoids and analyzed using high performance chromatography with photodiode array detection (HPLC-PDA). Phytoene, phytofluene, ζ-carotene, neurosporene, tetra-\textit{cis} lycopene, all-\textit{trans} lycopene and other \textit{cis}-lycopenes were quantified. Tetra-\textit{cis}-lycopene decreased with increasing heat time, decreasing by 80\% of the original level in sauce cooked for 180 min. Phytoene and phytofluene were heat stable throughout processing. Levels of all-\textit{trans}-lycopene and other \textit{cis}-lycopenes increased significantly with longer processing times. Total carotenoids and total lycopene decreased with increased heating times. This study suggests limiting thermal processing of \textit{tangerine} tomato products if delivery of tetra-\textit{cis}-lycopene is desirable.
Keywords: tangerine tomato, *Solanum lycopersicum*, tomato processing, lycopene, tetra-
cis-lycopene, prolycopene, cis-lycopene, isomerization
2.1 Introduction

Increased tomato consumption has been associated with a decrease risk for certain cancers, particularly prostate cancer (12,13). This association has led to research focused on understating the biological effects of lycopene, the primary carotenoid pigment in tomato fruits. Tomatoes provide approximately 80% of the lycopene consumed my Americans, although watermelon, pink grapefruit and papaya also contribute to dietary consumption (155). Lycopene contains 11 conjugated (and 2 unconjugated) double bonds and can theoretically form 1056 geometrical (cis/trans) isomers. However, steric hindrance favors the formation of those isomers that exist in the lowest energy state, and the true number of geometrical isomers is likely significantly less (25). Almost all lycopene from raw fruits exists in the all-trans configuration. However, lycopene in the blood and tissues of humans and animals consuming a lycopene containing diet is enriched in cis isomers, ranging from ~50-88% of total lycopene in cis configurations(156). This discrepancy brings question as to the biological role of cis isomers of lycopene.

*Tangerine* tomatoes are orange when ripe tomatoes that are rich in cis-lycopene. The recessive mutation *tangerine* results in the loss of function of carotenoid isomerase (CRTISO) causing accumulation of poly-cis-lycopene at the expense of all-trans-lycopene (33). These tomatoes have been studied as early as the 1940s where they were found to contain lycopene in a tetra-cis (7Z, 9Z, 7′Z, 9′Z)-configuration, also called prolycopene (157). The term cis and Z (of the German E/Z system for geometrical
isomers) to distinguish isomeric form are often used interchangeably but do not truly mean the same thing. *Cis* isomers have their substituents on the same side of a carbon-carbon double bond, while *Z* specifies the two groups of higher priority on the same side of a carbon-carbon bond. Insertion of four *cis* bonds leads to a hypsochromic shift of 35 nm below all-*trans*-lycopene, imparting these tomatoes with their characteristic ‘tangerine’ hue (157). *Tangerine* tomatoes are one of the few raw fruits or vegetables that accumulate *cis*-lycopene (158). As a result of altered carotenoid biosynthesis, *tangerine* tomatoes also accumulate the lycopene precursors phytoene, phytofluene, ζ-carotene and neurosporene (Figure 2.1) at levels much higher than conventional red tomatoes (159).
Figure 2.1 – Structures of major carotenoids in tangerine tomatoes.
Cis-lycopene isomers have been shown to be more bioavailable in humans (36). Recent work has shown that the same dose of lycopene is, on average, 8.5-fold more bioavailable from tangerine tomatoes than from red tomatoes (159). This marked increase in bioavailability has been attributed to the presence of cis-lycopene isomers, as well as lycopene deposited in non-crystalline lipid dissolved droplets within tangerine tomato chromoplasts (159). Cis-lycopenes (including tetra-cis-lycopene) also have higher antioxidant activities compared to all-trans-lycopene (160,161).

Lycopene bioavailability is greatly affected by thermal processing, with processed tomatoes being more bioavailable compared to raw (80). This is thought to be a function of increased liberation of lycopene from within the food matrix (80), in addition to isomerization of the lycopene to its more bioavailable cis forms (162). The presence of fat in a meal has also been shown to be critical for carotenoid absorption (90), while type of fat also plays a role (93).

The effects of thermal processing on carotenoid profiles in red tomatoes have been extensively studied. Lycopene from tomato products seems to be relatively stable to moderate heat processing, especially in the absence of fat (35,69). Extensive heat processing can cause degradation (68). It has been shown that lycopene is more stable in a tomato matrix, compared to isolated, purified or in solvents (70). The effects of fat on lycopene isomerization from tomatoes have yielded mixed results. The addition of 5% or 15% olive oil has been shown to not affect lycopene isomer profile (35) while tomato
juice heated in an oven at 180°C with 10% safflower showed significant isomerization (71). Schierle et al. demonstrated that tomato dissolved in water underwent less isomerization compared to tomato dissolved in oil olive, when subjected to 3 hrs of heating at 75 °C (72).

Different physical storage forms of lycopene in red and *tangerine* tomatoes likely contribute to differences in susceptibility to isomerization and degradation during thermal processing. Lycopene in red tomatoes is present as crystals, a relatively stable configuration (110,163). Lycopene in *tangerine* tomatoes is present in non-crystalline, lipid dissolved plastoglobules (108,159). This difference suggests that lycopene should be more stable to processing in red tomatoes, when it exists within the protection of crystalline structures.

Little research has been conducted to investigate the effects of different levels of thermal treatment and fat on a *tangerine* tomato product. The objective of this study is to investigate the effects that thermal processing and fat (both separately and combined) have on isomerization and content of lycopene and lycopene precursors in *tangerine* tomato juice and sauce, using high performance liquid chromatography with a photodiode array detector (HPLC-PDA).

2.2 Materials and methods
2.2.1 Chemicals

Acetone, hexane, methanol, methyl tert-butyl ether (MTBE) and were purchased from Fisher Scientific (Pittsburgh, PA) and solvents were of HPLC grade. Ammonium acetate was from J.T. Baker (Phillipsburg, NJ). Pure lycopene was isolated and crystallized from tomato paste for use in external standard curves as described previously (164).

2.2.2 Tomato juice/sauce processing

*Tangerine* tomato variety FG04-167 was grown and harvested at The Ohio State University’s (OSU) North Central Agriculture Research Station (Fremont, OH). After harvest, tomatoes were hot break processed at 88 °C into juice and canned in #10 cans at the OSU Food Industries Center (Columbus, OH). For juice experiments, *tangerine* tomato juice (5.1 °Brix, pH 4.3) was re-processed in 300x407 cans with 0, 1, 2 or 3 % olive oil (w/w), heated to 88 °C and held for 0, 30, 60 or 120 min in a duplicated 4x4 Latin square design. For sauce experiments, *tangerine* tomato juice was freeze dried to concentrate and powder was reconstituted with juice to 12.7 °Brix. Sauce was then re-processed with 0, 1, 5, 15 or 30 % olive oil (w/w) at 100 °C for 0, 30, 60, 120 or 180 min in a duplicated 5x5 Latin square design. Tomato products were processed without exposure to light and oxygen.

2.2.3 Carotenoid extraction

Carotenoids were extracted under red light from tomato juice and sauce using methods previously described (91). Briefly, ~1.5 g of juice or sauce was added to an 11 mL glass
centrifuge tube with 5 mL of methanol. Samples were probe sonicated (Digital Sonic Dismembrator 150 series, Fisher Scientific, Pittsburgh, PA) for 8 sec and placed on ice. Samples were centrifuged at 2000 x g for 5 min and supernatant was decanted and saved. Pellets were re-extracted with 5 mL of 1:1 hexane:acetone, probe sonicated for 8 seconds, and centrifuged for 5 min. Supernatant was decanted and samples were extracted 2 more times with 1:1 hexane:acetone or until pellet was colorless. Pooled extracts were phase separated with water, brought up to a known volume and aliquots were dried down and stored at -80 °C until further analysis. Samples were brought up in 1:1 MTBE:methanol for HPLC analysis.

2.2.4 HPLC-PDA analysis

Carotenoids were analyzed using reversed phase HPLC (Alliance 2965, Waters Corp., Milford, MA) and a PDA detector (996, Waters Corp.) and data was collected using Empower 2 (Waters Corp.). A 4.6 x 250 mm, 3 μm pore size C30 “carotenoid column” was used (YMC, Wilmington, NC). A gradient elution of solvent A: 88% methanol, 5% MTBE, 5% H2O, 2% aqueous ammonium acetate (2% w/v) and solvent B: 78% MTBE, 20% methanol, 2% aqueous ammonium acetate (2% w/v), with a flow rate of 1.3 mL/min and column temperature of 30 °C was used to separate carotenoids. A linear gradient was applied as follows: 45% B to 50% B over 20 min, to 95% B over the next 8 min, hold at 95% B for 4 min, to 100% B over 2 min, hold at 100% B for 3 min, followed by a reconditioning at initial conditions for 3 min.
2.2.5 Data analysis

External standard curves were created to quantify all-trans-lycopene (471 nm). Other carotenoids, including phytoene (286 nm), phytofluene (348 nm), ζ-carotene (400 nm), neurosporene (440 nm) and tetra-cis-lycopene (440 nm) were confirmed using spectral characteristics and mass spectrometry (QToF Premier, quadrupole time of flight mass spectrometer, Micromass UK Ltd., Manchester, United Kingdom) quantified using a ratio of each molar extinction coefficient compared to the molar extinction coefficient of all-trans-lycopene, to yield a relative slope. Other-cis-lycopenes (471 nm) were quantified as all-trans. Multivariate analysis was applied using thermal processing time and fat concentration as independent factors versus carotenoid content (SPSS v. 19, Chicago, IL) with Tukey’s HSD post-hoc test (α=0.05).

2.3 Results and discussion

2.3.1 Carotenoid content in tangerine tomato juice

Carotenoid content of the singly processed tangerine juice and sauce, as well as comparison to a typical red tomato is listed in Table 2.1. Tetra-cis-lycopene and other-cis-lycopene isomers (excluding tetra-cis) make up about 61% and 33% respectively of the total lycopene in tangerine tomato juice and sauce, with only about 6% of lycopene existing in the all-trans configuration. This profile of lycopene is consistent with other studies of tangerine tomato juices conducted by our group (159). Phytoene and ζ-carotene are the carotenoids present in highest concentration in tangerine tomatoes, and
exist in much lower concentrations in red tomatoes. *Tangerine* tomatoes contain about 30% of the total lycopene found in red tomatoes but have about two times the total carotenoid found in red tomatoes, as a result of high concentrations of lycopene precursors. Production of *tangerine* tomato sauce concentrated carotenoids approximately linearly in relation to soluble solids (measured using °Brix). In other words, *tangerine* tomato sauce was about 2.5 concentrated according to °Brix measurements, and the concentration of carotenoids in the *tangerine* tomato sauce is approximately 2.5 times higher. This is expected as the sauce was freeze dried as a method of concentration.
Table 2.1 - Carotenoid content (wet weight) in singly processed *tangerine* tomato sauce and juice, and comparison to a typical red tomato juice.

<table>
<thead>
<tr>
<th>Carotenoid</th>
<th>Mean <em>tangerine</em> tomato sauce carotenoid content (mg/100g)</th>
<th>Mean <em>tangerine</em> tomato juice carotenoid content (mg/100g)</th>
<th>Typical red tomato juice carotenoid content (mg/100g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phytoene</td>
<td>18.35</td>
<td>7.61</td>
<td>0.17</td>
</tr>
<tr>
<td>Phytofluene</td>
<td>6.13</td>
<td>2.55</td>
<td>0.23</td>
</tr>
<tr>
<td>ζ-carotene</td>
<td>13.63</td>
<td>5.77</td>
<td>ND</td>
</tr>
<tr>
<td>Neurosporene</td>
<td>2.87</td>
<td>1.16</td>
<td>ND</td>
</tr>
<tr>
<td>Tetra-cis-lycopene</td>
<td>2.77</td>
<td>1.34</td>
<td>ND</td>
</tr>
<tr>
<td>Other-cis-LYC</td>
<td>1.72</td>
<td>0.72</td>
<td>0.11</td>
</tr>
<tr>
<td>All-trans-lycopene</td>
<td>0.36</td>
<td>0.13</td>
<td>6.81</td>
</tr>
<tr>
<td>Total lycopene</td>
<td>4.85</td>
<td>2.19</td>
<td>6.92</td>
</tr>
<tr>
<td>β-carotene</td>
<td>ND</td>
<td>ND</td>
<td>0.17</td>
</tr>
<tr>
<td>Lutein</td>
<td>ND</td>
<td>ND</td>
<td>ND*</td>
</tr>
<tr>
<td>Total carotenoid</td>
<td>45.83</td>
<td>20.62</td>
<td>7.49</td>
</tr>
</tbody>
</table>

ND: not detected

*some red tomatoes contain small quantities of lutein
A representative chromatogram of *tangerine* tomato sauce is shown in Figure 2.2. Chromatograms for *tangerine* tomato juice have similar separations with lower carotenoid concentrations. The structural similarity of the carotenoids in *tangerine* tomatoes make for challenging chromatography. Most *tangerine* tomato carotenoids can distinguished, if separated, by their unique spectral characteristics (165). Neurosporene and, closely eluting (likely tri- and/or di-*cis*-) lycopene isomers have similar spectral features but can be distinguished using mass spectrometry because of a mass difference of 2 amu. All peaks identities were verified using mass spectrometry.
Figure 2.2 - HPLC-PDA chromatogram of tangerine tomato sauce. Panels A-E are extracted at 286, 348, 400, 440 and 471 nm respectively. Peak identification: 1: phytoene, 2a-b: phytofluene, 3a-c: ζ-carotene, 4: tetra-cis-lycopene, 5a-c: neurosporene, 6a-g: cis-lycopene isomers, 6h: all-trans-lycopene, 6i: 5-cis-lycopene
2.3.2 Processing effects on lycopene and lycopene isomers

Tetra-cis-lycopene significantly decreased in tangerine tomato sauce with each additional increment of heating duration (Figure 2.3). By 180 min of heating, tetra-cis-lycopene has decreased ~80% compared to sauce heated for 0 min. With increased heating time, all-trans-lycopene and other-cis-lycopene significantly increases while total lycopene decreases. This suggests that tetra-cis-lycopene is isomerizing to other-cis forms, as well as to all-trans. The magnitude of this conversion is unknown since total lycopene decreases over the 180 min. We hypothesize that there is isomerization from tetra-cis-lycopene, to other cis forms, in addition to degradation and/or oxidation to compounds that were unable to be identified here. No significant differences were found in carotenoid content between fat levels processed for the same amount of time. Similar trends were found in the tangerine tomato juice (data not shown) however the differences were of smaller magnitude, likely a function of a less severe heat process.
Figure 2.3 – Tetra-cis-lycopene (A), other-cis-lycopene (B), all-trans-lycopene (C) and total lycopene (D) content of tangerine tomato sauce processed at 100°C for 0, 30, 60, 120 or 180 minutes with 0, 1, 5, 15 or 30% fat. Black bars are 0% fat, black/white striped bars are 1% fat, grey bars are 5% fat, grey dotted bars are 15% fat and white bars are 30% fat. Different letters denote statistically significant differences between heat treatments using Tukey’s post-hoc test ($P < 0.05$). No significant differences exist between fat levels.
These data are consistent with those of Hackett et al. which found that the rate of degradation of lycopene was higher in *tangerine* compared to red tomatoes (166). Nguyen et al. concluded that unlike β-carotene, lycopene (from red tomatoes) is quite resistant to isomerization during processing of food products. Often, the conditions that are used in the food industry are not harsh enough to form high levels of *cis*-lycopene isomers (35).

Lycopene within a tomato matrix appears to be much more resistant to thermal degradation/isomerization as compared to once it is extracted and dissolved in solvents (167). This matrix protection may be a result of crystalline lycopene present in a stable configuration, not solubilized with resistance to degradation and isomerization. Since lycopene in *tangerine* tomatoes is present in lipid dissolved droplets, this protection is absent. This leads to lycopene being sensitive to isomerization and degradation in *tangerine* tomato products.

### 2.3.3 Processing effects on phytoene, phytofluene, ζ-carotene and neurosporene

Phytoene and phytofluene did not significantly differ from initial after 180 minutes of boiling (Figure 2.4). Graziani et al., found decreases in phytoene and phytofluene over 9 hours of boiling in peeled red tomatoes but no real changes over heating when processing times were equivalent to those in study (168). Additionally, Takeoka et al. also didn’t find significant differences in phytoene, phytofluene and ζ-carotene during processing of fresh red tomatoes into hot break juice and paste (169).
Figure 2.4 – Phytoene and phytofluene content of *tangerine* tomato sauce processed at 100°C for 0, 30, 60, 120 or 180 minutes with 0, 1, 5, 15 or 30% fat. Black bars are 0% fat, black/white striped bars are 1% fat, grey bars are 5% fat, grey dotted bars are 15% fat and white bars are 30% fat. No statistically significant differences between heat treatments using Tukey’s post-hoc test were found between any of the heating times. No significant differences exist between fat levels.
Traditionally, it was thought that phytoene and phytofluene exist mostly in the 15-cis configuration while ζ-carotene is primary all-trans, with smaller amounts of other cis-isomers (170). Others have proposed that in *tangerine* tomatoes, phytoene, phytofluene, ζ-carotene and neurosporene all occur as cis-isomers (33). Surprisingly, phytoene and phytofluene were resistant to degradation despite their existence in cis configurations which are theoretically less stable. We have found in our lab that phytoene and phytofluene purchased as standards are extremely sensitive to degradation when dissolved in solvents. This suggests that something about the matrix of a tomato product protects these colorless carotenoids during heating, more so than when they are dissolved in organic solvents.

Phytoene, phytofluene and ζ-carotene have been found in appreciable concentrations in blood, human milk and other tissues (171,172). Phytoene seems to be enriched in lung (4 times the levels of lycopene) and skin (equal concentration to lycopene) despite being present in the diet is lower concentrations (172). Neurosporene has also been found in human ciliary bodies (173). Preferential deposition of these carotenoids suggests potential differential biological roles *in vivo*.

ζ-carotene and neurosporene also significantly decreased after 180 minutes of heating, but the magnitude of this decrease is much less compared to the decreased noted with tetra-cis-lycopene (Figure 2.4). Little information exists in the literature on the effects of
thermal processing on ζ-carotene and neurosporene, likely because they exist in low concentrations in most foods.

Processing in red tomatoes can increase extractability of carotenoids. In red tomatoes, the lycopene takes time to dissolve from its crystalline state, thus giving an apparent increase in levels after heating. This was not noted here, likely because of the physical deposition form of these carotenoids in tangerine tomatoes. Since carotenoids in tangerine tomatoes are not crystalline, they are less protected from thermal degradation than one might observe with red tomatoes. Even in processed red tomato products, lycopene have been shown to be present in crystals (35,174), suggesting that lycopene physical form may be providing some of the noted stability to thermal processing.

Little is known about how food processing affects the bioavailability of phytoene, phytofluene, ζ-carotene and neurosporene. We hypothesize that thermal processing will not increase bioavailability for these lycopene precursors in the same way that it does for lycopene since these carotenoids already exist in cis configurations and are likely present in non-crystalline forms. As a result, heating does not liberate insoluble carotenoid making it more bioaccessible and therefore more bioavailable.

2.4 Conclusions

Overall, this was the first study investigating the effects of different thermal processing times on carotenoids in tangerine tomato products. Tetra-cis-lycopene from tangerine
tomatoes is heat labile and degrades up to 80% after three hours of thermal processing. Phytoene and phytofluene within a tomato matrix are stable to thermal processing. This research suggests that, if delivery of tetra-cis-lycopene from *tangerine* tomatoes is desirable, thermal processing should be minimized.
CHAPTER 3 - ENHANCED BIOAVAILABILITY OF LYCOPENE IN HUMANS WHEN CONSUMED AS CIS-ISOMERS FROM TANGERINE TOMATOES COMPARED TO RED TOMATOES

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Abbreviations: AUC, area under the curve; BCO1, β-carotene oxygenase 1; C_max, concentration maximum; CRTISO, carotenoid isomerase; CRC, The Ohio State University’s Clinical Research Center; IRB, Institutional Review Board; MTBE, methyl tert-butyl ether; OSU, The Ohio State University; PBMC, peripheral blood mononuclear cells; TEM, transmission electron microscopy; TGF-β1, tumor growth factor β1; TRL, triglyceride-rich lipoprotein fraction of plasma

Key words: bioavailability, carotenoids, chromoplast, tangerine tomato, tetra-cis-lycopene

Modified from submission to Molecular Nutrition and Food Research
3.0 Abstract

**Scope:** *Solanum lycopersicum* fruit that are rich in tetra-cis-lycopene due to natural variation in carotenoid isomerase are referred to as tangerine tomatoes. Our objective was to compare the bioavailability of lycopene from tangerine compared to red tomato juice in humans and to elucidate the physical deposition form of these isomers by light and electron microscopy.

**Methods and results:** Following a randomized, two-way crossover design, subjects (*n* = 11) consumed two meals with 10 mg lycopene from tangerine or red tomato juice. Blood was sampled over 12 hours and triglyceride-rich lipoprotein fractions of plasma (TRLs) were isolated and analyzed using HPLC-DAD-MS/MS. Lycopene was better absorbed from tangerine tomatoes compared to red (*P* < 0.001), with an average difference of 8.5-fold among all subjects and 52-fold within an individual. Large heterogeneity was observed among subjects. Lycopene was crystalline in red tomato chromoplasts and globular in tangerine tomatoes. No differences were noted in inflammatory markers/cytokines after one tomato containing meal.

**Conclusions:** Lycopene was markedly more bioavailable from tangerine than from red tomatoes, consistent with a predominance of cis-lycopene isomers and presence in chromoplasts in a lipid dissolved globular state. These results justify using tangerine tomatoes as a lycopene source when formulating functional foods with potential health benefits.
3.1 Introduction

Epidemiological evidence suggests that diets rich in tomatoes and tomato products may be protective against risk for certain cancers, especially prostate cancer (12). Many in vitro, in vivo and human clinical studies support this hypothesis. The carotenoid lycopene has received the most attention as the compound in tomatoes responsible for this noted decrease in cancer risk. In raw, red tomatoes, approximately 95% of the total lycopene is present in the all-trans form (26). Despite the predominance of dietary all-trans-lycopene, cis-isomers account for 58-73% of total lycopene in human serum, and a surprisingly high 79-88% in benign or malignant prostate tissue (27). This apparent discrepancy has been partly related to the preferential uptake of cis-lycopene during digestion. Compared to the all-trans forms, cis-lycopene isomers are less likely to crystallize, more oil/hydrocarbon soluble, (29) preferentially micellarized (30), and more readily taken up by intestinal cells (31). All-trans-lycopene was also shown to isomerize to cis-forms in vivo using $^{14}$C (175) and $^{13}$C tracer approaches (176).

In contrast to red tomatoes, tangerine tomatoes are a unique cis-lycopene rich type of tomato developed through conventional breeding techniques (Figure 1). These tomatoes have the recessive mutation tangerine (32) and lack a functional form of the enzyme carotenoid isomerase (CRTISO), which converts poly-cis- into all-trans-lycopene and is necessary for the biosynthesis of downstream cyclized carotenoids (33). As a result, tangerine tomatoes accumulate several cis-lycopenes, with tetra-cis-lycopene ((7Z, 9Z,
7′Z, 9′Z)-lycopene, also called prolycopene) predominating at the expense of the all-trans form. Tetra-cis-lycopene absorbs light maximally approximately 35 nm below all-trans-lycopene, resulting in tomatoes with an orange color as shown in Figure 1 (34). Most processed foods containing tomato have concentrations of cis isomers less than 10% (35) although severe food processing conditions can increase the percentage of cis-lycopenes, shown to increase bioavailability (36). Stemming from this result, others have suggested tetra-cis-lycopene may be more bioavailable than all-trans (37).

Tangerine tomatoes also contain considerable levels of phytoene, phytofluene, ζ-carotene and neurosporene, in addition to other mono-, di- and tri-cis-lycopene isomers in comparison to all-trans-lycopene containing red tomatoes. It is hypothesized that other carotenoids upstream in the biosynthesis of tomato products, especially phytoene and phytofluene, may also play a role in disease prevention [15, 16]. Additionally, there is evidence that prolonged tomato consumption can influence markers of oxidative stress [17, 18] and inflammation (177), while consuming tomatoes with even a single high-fat meal modulating post-prandial oxidative stress and related inflammation (66).

In order to manifest their potential beneficial health effects, carotenoids must be liberated from the food matrix and subsequently solubilized into mixed micelles before they can be absorbed. In common red tomatoes, lycopene is found in large crystalline aggregates of up to 15 µm length within chromoplasts, the cellular organelle where carotenoids are biosynthesized and deposited (105). Carotenoid liberation and solubilization from such
crystals was hypothesized to be significantly lower when compared to lycopene from smaller aggregates [23]. This suggests that chromoplast morphology may play a role in post-prandial bioavailability of carotenoids. Non-crystalline deposition of lycopene is rarely found in natural plant foods since all-trans-lycopene easily crystallizes. Therefore, most common lycopene containing fruits (red tomatoes, watermelon, and red-fleshed papaya) have crystalline lycopene aggregates (105,108). Often, in order to have lipid-dissolved carotenoids a high concentration of fat is required, as previously reported for carotenoids in peach palm (*Bactris gasipaes* Kunth) fruits (111). Although *tangerine* tomato contains only minor amounts of fat, tetra-cis- and other cis-isomers are expected to be deposited in a non-crystalline form. A lipid-dissolved deposition state within small lipid globules (plastoglobules) of *tangerine* chromoplasts has been suggested (108), although simultaneous investigations of the carotenoid profile and the chromoplast ultrastructure has not been conducted. Due to the importance for bioavailability, the first goal was to compare the carotenoid profiles and chromoplast ultrastructures of our red and *tangerine* tomatoes.

The main objective of this study was to evaluate, on an equal dose basis, the bioavailability of lycopene from *tangerine* tomato juice compared to red tomato juice in humans. This approach allows a direct comparison of the bioavailability of cis- vs trans-lycopene from a natural source. In addition, it allows inferences to be made about the relationship between lycopene cis isomer composition in conjunction with carotenoid chromoplast morphology and its resulting effect on bioavailability in humans. In
addition, we investigated whether the consumption of one tomato-rich meal by healthy subjects can modulate markers of inflammation \textit{in vivo}.

\section*{3.2 Materials and methods}

\subsection*{3.2.1 Materials}

All solvents and NaCl were obtained from Fisher Scientific (Pittsburgh, PA). Acetone and methyl tert-butyl ether (MTBE) were HPLC grade and methanol and water were Optima grade. Ammonium acetate was purchased from J.T. Baker (Phillipsburg, NJ, USA). Lycopene was isolated and crystallized from tomato paste as previously described (164). Phytoene, phytofluene, \(\zeta\)-carotene, neurosporene and tetra-cis-lycopene were isolated from \textit{tangerine} tomato extracts using preparative HPLC. Identity and purity (>95\%) was confirmed with HPLC/accurate mass before using as an external calibrant.

\subsection*{3.2.2 Subjects}

A total of 11 subjects completed both intervention arms of this clinical trial. Volunteers (6 male, 5 female) were healthy, non-pregnant, non-smoking, normocholesterolemic (<200 mg/dL), normolipidemic (<200 mg/dL), hemoglobin and hematocrit levels at or above 10 g/dL and 30\% respectively, and had BMIs between 18.5-30 kg/m\(^2\). Additionally, subjects were free of any metabolic disease (including diabetes mellitus or thyroid dysfunction), malabsorption disorders (including ileus, Crohn’s disease, ulcerative colitis and pancreatic insufficiency), history of cancer, esophageal, gastric or
intestinal ulcers, history of liver or kidney insufficiency/failure, autoimmune disorders, chronic inflammatory syndromes (including rheumatoid arthritis) and allergies to tomatoes or tomato products. Baseline subject characteristics can be found in Table 1. At the initial visit, subjects were screened to ensure they met inclusion criteria using a Dimension Xpand Plus Automated Clinical Chemistry Analyzer (Siemens, New York, NY) and LH 780 hematology analyzer (Beckman Coulter, Brea, CA).

Informed consent was obtained from all subjects prior to any study activity and all study procedures were performed at The Ohio State University’s (OSU) Clinical Research Center (CRC). This study was approved by the OSU Institutional Review Board (IRB, protocol #2012H0189), the CRC (Clinical Center for Translational Science ID #1995) and registered with ClinicalTrials.gov (NCT01696773).
Table 3.1 - Baseline subject characteristics at enrollment

<table>
<thead>
<tr>
<th>Sex</th>
<th>Age (years)</th>
<th>BMI (kg/m²)</th>
<th>Plasma total cholesterol (mg/dL)</th>
<th>Plasma triglycerides (mg/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>All subjects ($n = 11$)</td>
<td>26.5 ± 10.1</td>
<td>23.3 ± 1.49</td>
<td>174 ± 11.1</td>
<td>77.9 ± 46.6</td>
</tr>
<tr>
<td>Female ($n = 5$)</td>
<td>29.2 ± 14.9</td>
<td>22.7 ± 1.23</td>
<td>182 ± 9.53</td>
<td>70.4 ± 53.9</td>
</tr>
<tr>
<td>Male ($n = 6$)</td>
<td>24.2 ± 3.54</td>
<td>23.8 ± 1.62</td>
<td>167 ± 7.20</td>
<td>84.2 ± 43.8</td>
</tr>
</tbody>
</table>
3.2.3 Study design

This study was conducted using a randomized, two-way cross-over design. All subjects were provided with a list of lycopene-containing foods to avoid and were instructed to consume a low-lycopene diet for 14 days prior to each day-long clinic visit to sufficiently washout and avoid carryover of carotenoids from previous meals (94). Subjects refrained from consuming any foods with more than 1 mg/serving of lycopene, according to the USDA Nutrient Database and Standard Reference Release 25. Subjects were instructed to fast overnight (12 hr) prior to their first day-long visit. After drawing a sample of baseline blood (0 hr) via lower arm catheter into glass, EDTA vacutainer® tubes (BD and Co., Franklin Lakes, NJ) subjects were instructed to consume their tomato juice containing breakfast within 20 min. Blood was then taken at 2, 3, 4, 5, 6, 8, 10 and 12 hrs with lunch at 4.5 hrs. No snacks were permitted during the 12-hr collection period and subjects were allowed to consume water ad libitum. Subjects then left the clinic, continued their washout diet for the next 14 days and returned to the clinic for their second day-long visit to consume the test meal with the tomato juice they did not consume on their first day-long visit.

3.2.4 Tomato juices and study meals

_Tangerine_ tomatoes (*Solanum lycopersicum* L. hybrid FG10-314) and red tomatoes (*Solanum lycopersicum* L., hybrid derived from OH8245xOH8243) were grown at the OSU’s North Central Agriculture Research Station, Fremont, OH. These tomatoes were
harvested, processed into juice, salted (7.4 g NaCl/L juice) and hot filled at 93 °C into cans in the OSU Food Industries Center Pilot Plant (Columbus, OH).

Tomato juices were provided in conjunction with a breakfast at the CRC. Breakfast contained either red or tangerine tomato juice providing 10 mg of total lycopene, 10 g of canola oil, 1 English muffin (57 g), 2 large egg whites (66 g) scrambled without any fat, 1 banana (118 g), fat-free vanilla Greek yogurt (170 g), honey (14 g) and coffee (356 g) with non-fat creamer and sugar. Breakfast provided 521 kcal, 30 g protein, 11.7 g fat, 77 g carbohydrates and 5.7 g fiber. A very low-fat lunch was provided at 4.5 hours which included fat-free multigrain bread (52 g), fat-free turkey breast (90 g), fat-free Swiss-style cheese (42 g), 1 banana (118 g), fat-free pretzels (56 g), canned white peaches (166 g), cauliflower (62 g) cooked without fat and fat-free mayonnaise-like spread (5.3 g). Lunch provided 692 kcal, 42 g protein, 1.6 g fat, 127 g of carbohydrates and 7.8 g fiber. Values were calculated using the Nutrient Data System for Research software (University of Minnesota, Minneapolis, MN). Neither breakfast nor lunch contained appreciable carotenoids, except for the test tomato juice.

3.2.5 Triglyceride-rich lipoprotein fraction of plasma (TRL) isolation and carotenoid extraction

TRLs were isolated using slight modifications from trial 1 of Kopec et al. (178) using a SW 55 Ti swinging bucket rotor and an Optima L-100XP ultracentrifuge (Beckman
Coulter, Brea, CA). Carotenoids were extracted from tomato juices and TRLs as previously described by Kopec et al. (178).

3.2.6 Carotenoid analysis by HPLC-DAD and HPLC-DAD-MS/MS

Carotenoids from tomato juices were analyzed using HPLC-DAD (Alliance 2695, 996 DAD, Waters Corporation, Milford, MA) and TRL extracts were analyzed using HPLC-DAD-MS/MS (Agilent 1260, Santa Clara, CA, interfaced with an AB Sciex QTrap 5500 mass spectrometer, Foster City, CA). Analytes were separated on a C30 column (4.6x250 mm, 3 μm, YMC Inc., Wilmington, NC) at 35 °C using a gradient of A: 60% methanol, 35% MTBE, 3% water, 2% aqueous ammonium acetate (2% w/v), and B: 78% MTBE, 20% methanol, 2% aqueous ammonium acetate (2% w/v) flowing at 1.3 mL/min. A linear gradient was applied as follows: 0% B to 35.6% B over 9 min, to 100% B over the next 6.5 min, hold for 3.5 min at 100% B, and equilibrate for 3.5 min at initial conditions. Tomato juice extracts were re-dissolved in 2 mL of 1:1 MTBE:methanol, filtered using a 13 mm, 0.2 μm pore nylon filter, and 10 μL was injected. TRL extracts were re-dissolved in 200 μL 1:1 MTBE:methanol, centrifuged (model 5424, Eppendorf, Hamburg, Germany) at 21,130 x g for 2 min, and 20 μL of the supernatant was injected. Phytoene, phytofluene and ζ-carotene were quantified using DAD while neurosporene and all lycopene isomers were quantified using MS/MS. HPLC-DAD-MS/MS parameters are shown in Table 2.
Table 3.2 - HPLC-PDA-MS/MS parameters for carotenoid analysis of TRLs.

<table>
<thead>
<tr>
<th>Peak label</th>
<th>Compound identity</th>
<th>HPLC-PDA $\lambda_{\text{max}}$ (nm)</th>
<th>HPLC-APCI(+)-MS/MS experiments</th>
<th>CE$^a$ (eV)</th>
<th>Dwell$^b$ (msec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>phytoene</td>
<td>286</td>
<td>545.51&gt;463.6, 421.6, 395.6, 327.4</td>
<td>22, 22, 29, 25</td>
<td>165</td>
</tr>
<tr>
<td>2</td>
<td>phytofluene isomer</td>
<td>348</td>
<td>543.49&gt;461.6, 393.6, 325.4</td>
<td>29, 29, 22</td>
<td>165</td>
</tr>
<tr>
<td>3</td>
<td>phytofluene isomer</td>
<td>348</td>
<td>543.49&gt;461.6, 393.6, 325.4</td>
<td>29, 29, 22</td>
<td>165</td>
</tr>
<tr>
<td>4</td>
<td>$\alpha$-carotene</td>
<td>445</td>
<td>537.45&gt;455.3, 269.2</td>
<td>22, 20</td>
<td>135</td>
</tr>
<tr>
<td>5</td>
<td>tetra-cis-lycopene</td>
<td>439</td>
<td>537.45&gt;455.3, 269.2</td>
<td>22, 20</td>
<td>135</td>
</tr>
<tr>
<td>6</td>
<td>neurosporene</td>
<td>410</td>
<td>539.45&gt;457.6, 415.4, 389.6</td>
<td>22, 29, 29</td>
<td>135</td>
</tr>
<tr>
<td>7</td>
<td>$\zeta$-carotene isomer</td>
<td>400, 425</td>
<td>541.47&gt;391.4, 349.7, 271.27</td>
<td>22, 22, 20</td>
<td>135</td>
</tr>
<tr>
<td>8</td>
<td>$\beta$-carotene</td>
<td>451</td>
<td>537.45&gt;455.3, 269.2</td>
<td>22, 20</td>
<td>135</td>
</tr>
<tr>
<td>9</td>
<td>$\zeta$-carotene isomers</td>
<td>400, 425</td>
<td>541.47&gt;391.4, 349.7, 271.27</td>
<td>22, 22, 20</td>
<td>135</td>
</tr>
<tr>
<td>10, 11</td>
<td>neurosporene isomers</td>
<td>440</td>
<td>539.45&gt;457.6, 415.4, 389.6</td>
<td>22, 29, 29</td>
<td>180</td>
</tr>
<tr>
<td>12</td>
<td>other-cis-lycopenes</td>
<td>440-471</td>
<td>537.45&gt;455.3, 269.2</td>
<td>22, 20</td>
<td>180</td>
</tr>
<tr>
<td>13</td>
<td>all-trans-lycopene</td>
<td>471</td>
<td>537.45&gt;455.3, 269.2</td>
<td>22, 20</td>
<td>180</td>
</tr>
<tr>
<td>14</td>
<td>5-cis-lycopene</td>
<td>471</td>
<td>537.45&gt;455.3, 269.2</td>
<td>22, 20</td>
<td>180</td>
</tr>
</tbody>
</table>

$^a$CE: collision energy, $^b$multiple reaction monitoring experiments were sorted into periods to maximize dwell times for each analyte. All analytes were run with the following parameters: declustering potential: 185 V, entrance potential: 10 V, collision cell exit potential: 11 V, curtain gas: 30 psi and source temperature: 450 °C. Neurosporene and all lycopene isomers were quantified using the sum of the MS/MS transitions. Chromatogram is provided in Figure 4.
3.2.8 Microscopy

Light microscopy was performed as previously described by Schweiggert et al. (105). Free hand sections of tangerine and red tomato mesocarp close to the skin were viewed without staining on a Leica DM IRB microscope (Buffalo Grove, IL) equipped with a Q Imaging Retiga 2000 (Surrey, British Columbia, Canada). Transmission electron microscopy (TEM) sample preparation was performed according to Schweiggert et al. (105). Raw tomato samples were viewed using a Hitachi H-7500 TEM (Hitachi High-Tech, Toyko, Japan) at 80 kV. Photoshop CS4 (Adobe Systems, San Jose, CA) was used to adjust contrast and brightness of the micrographs.

3.2.9. Procurement of peripheral blood and preparation of RNA for inflammatory assays

Approximately 8-10 mL of blood was drawn from subjects following informed consent under and IRB approved protocol into sodium heparin tubes at baseline and 6 hrs following the experimental meal. Peripheral blood mononuclear cells (PBMCs) were separated from whole blood using Ficoll-Paque (Amersham Pharmacia Biotech, Uppsala, Sweden) via density gradient centrifugation as previously described (179). Cell pellets were resuspended in 1 mL of TRIzol (Life Technologies, Inc., Carlsbad, CA) and total cellular RNA was extracted as recommended by the manufacturer. This RNA was used as a template for Gene Expression Analysis via the NanoString-nCounter™ based quantification method.
3.2.10 Gene expression analysis

Gene expression analysis was performed using the NanoString-nCounter™ based gene quantification method. Probes specifically targeting the desired genes of interest were designed and manufactured by Nanostring Technologies (Seattle, WA) and analyses were performed by the Genomics Shared Resource laboratory at The Ohio State University. A codeset specific to a 100-base region of the target mRNA was designed using a 3′ biotinylated capture probe and a 5′ reporter probe tagged with a specific fluorescent barcode; creating two sequence-specific probes for each target transcript. Because of the expected low level of expression of the cytokines being analyzed, 1000 ng of total RNA for each sample was used for hybridization and the high sensitivity protocol was used on the nCounter Digital Analyzer.

Each codeset included probes for 22 cytokines, 5 reference housekeeping genes, and spiked-in positive and negative controls. Raw data files were imported into nSolver™ analysis software using NanoString® raw code count collector tool. Background hybridization was determined using spiked-in negative controls. All signals below mean background plus 2 SD were considered to be below the limits of detection, and set to mean background. A normalization factor was calculated from the spiked in exogenous positive controls in each sample and applied to the raw counts from the nCounter™ output data. Then, a content normalization factor was calculated from the Geomean of the reference genes and applied to the data previously normalized by the positive control.
Fold differences were calculated by dividing the normalized count for the 6 hr time point by the normalized counts for the 0 hr time point, for each visit separately.

3.2.11 Statistical and data analyses

Statistical analysis was performed using SPSS version 21 (IBM, Armonk, NY). Baseline correct AUC over 12 hours was derived using trapezoidal approximation. AUC for total lycopene from *tangerine* and red tomato juices were compared using Student’s *t*-test, and significance reported at *P*<0.05. Fractional absorption was calculated using the following equation:

\[
\text{Fractional absorption} = (\ln 2/t_{1/2}) \times \left[ \frac{(\text{AUC}_{\text{lycopene}} \times \text{MW}_{\text{lycopene}} \times \text{plasma volume})}{\text{dose}_{\text{lycopene}}} \right]
\]

This calculation assumes that the half-life (*t*\(_{1/2}\)) of lycopene is equal to that of chylomicrons (0.192 h) and plasma volume (mL) is equal to 927 + (31.47*body weight in kg) [36, 37].
3.3 Results and discussion

3.3.1 Carotenoid analysis of tomato juices

Carotenoid content in the tangerine and red tomato juices as dosed are listed in Table 3. The tangerine and red tomato juices each contained 10 mg of total lycopene, a dose achievable through diet alone. The red tomato juice provided approximately 90% of the lycopene in the all-trans- configuration while the tangerine tomato juice provided 94% of the lycopene in cis configurations. Tetra-cis-lycopene made up 58% of the lycopene from the tangerine tomato juice with an additional 36% as mono-, di- and tri-cis-lycopene. This distribution allows us to compare the absorption of cis vs. trans lycopene, both within a tomato matrix. In addition to providing lycopene, both juices contain phytoene and phytofluene (although in higher concentration in the tangerine tomato juice), and the tangerine tomato juice had ζ-carotene and neurosporene.
Table 3.3 - Carotenoid mean values in test meals as determined using HPLC-PDA.

<table>
<thead>
<tr>
<th>Carotenoid</th>
<th>Tangerine tomato juice&lt;sup&gt;a&lt;/sup&gt; (mg)</th>
<th>Red tomato juice&lt;sup&gt;b&lt;/sup&gt; (mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phytoene</td>
<td>44.9 ± 0.42</td>
<td>2.20 ± 0.06</td>
</tr>
<tr>
<td>Phytofluene</td>
<td>13.9 ± 0.16</td>
<td>0.69 ± 0.04</td>
</tr>
<tr>
<td>ζ-carotene</td>
<td>29.3 ± 1.81</td>
<td>nd</td>
</tr>
<tr>
<td>Neurosporene</td>
<td>6.26 ± 0.20</td>
<td>nd</td>
</tr>
<tr>
<td>Tetra-cis-lycopene</td>
<td>5.80 ± 0.09</td>
<td>nd</td>
</tr>
<tr>
<td>Other-cis-lycopene</td>
<td>3.64 ± 0.05</td>
<td>1.00 ± 0.25</td>
</tr>
<tr>
<td>All-trans-lycopene</td>
<td>0.57 ± 0.05</td>
<td>9.0 ± 0.31</td>
</tr>
<tr>
<td>Total lycopene</td>
<td>10.0 ± 0.16</td>
<td>10.0 ± 0.33</td>
</tr>
<tr>
<td>β-carotene</td>
<td>ND</td>
<td>0.07 ± 0.001</td>
</tr>
<tr>
<td>Lutein</td>
<td>ND</td>
<td>0.01 ± 0.003</td>
</tr>
<tr>
<td>Total carotenoid</td>
<td>104.3 ± 2.96</td>
<td>13.1 ± 0.32</td>
</tr>
</tbody>
</table>

<sup>a</sup>Values are reported as means ± SD in 505 g of tangerine tomato juice, <i>n</i> = 4

<sup>b</sup>Values are reported as means ± SD in 94 g of red tomato juice, <i>n</i> = 4

nd: not detected
3.3.2 Carotenoid physical deposition forms in red and tangerine tomatoes

Light microscopy and TEM revealed stark differences between the two types of tomato fruits. In Figure 1, the typical needle-shaped elongated chromoplasts of red tomato fruit are depicted (B1), containing large crystalline aggregates of the predominant red pigment, all-trans-lycopene. These elongated chromoplasts are absent in tangerine tomato mesocarp and much smaller, round chromoplasts were observed by light microscopy (Figure 1, B2). The crystalline deposition form of all-trans-lycopene in red tomatoes has been reported frequently in literature (105,108,110,182,183) and, in agreement, our transmission electron micrographs show typical all-trans-lycopene crystal remnants with characteristic electron-dense, undulated internal structures (Figure 2A). Tangerine tomato chromoplasts were devoid of these characteristics elements, instead containing numerous plastoglobules as the only carotenoid-bearing element according to the classification of Sitte et al. (108).
Figure 3.1 - Photographs of red (A1) and tangerine (*Solanum lycopersicon* L. hybrid FG10-314) tomatoes (A2) with corresponding light micrographs at 400x magnification of fresh red tomato (B1) and *tangerine* tomato (B2) mesocarp. Arrows and arrowheads denote crystalline and non-crystalline carotenoid containing structures, respectively.
As shown in Figure 3B and C, these plastoglobules were different in size and frequently contained an electron-dense surface area, potentially representing a lipoprotein layer (184). According to these observations, the cis-lycopene of our tangerine tomato variety is deposited in a lipid-dissolved physical state. This finding is consistent with previous work on Golden Jubilee tomatoes, a variety reported to contain the tangerine gene and similar globular chromoplasts (108). Globular chromoplasts containing high amounts of carotenoids were previously observed in comparatively lipid-rich fruits such as peach palm (*Bactris gasipaes* Kunth) fruits, which contain enough lipids to dissolve all present carotenoids (111). Since *tangerine* tomato does not contain appreciable lipid, the cis-lycopene itself might be responsible for the oily aggregate form. A similar carotenoid deposition was reported in globular structures of the microalgae *Dunaliella* sp., which contains extremely high concentrations (up to 10% of the dry weight) of all-trans-β-carotene and its 9-cis-isomer (185). Ben-Amotz et al. concluded that the presence of ca. 40% of the 9-cis-isomer of total β-carotene might help maintaining the oily rather than a crystalline deposition form of all *Dunaliella* carotenoids. Supporting this hypothesis, the visual appearance of the plastoglobules in *tangerine* tomato was different as compared to those of red tomato chromoplasts (Figure 3), potentially indicating a compositional difference. As described below, naturally different chromoplastidal deposition forms have previously been hypothesized to substantially influence carotenoid bioavailability.
Figure 3.2 - Transmission electron micrographs of fresh red tomato (A) and *tangerine* tomato (B, C) mesocarp. Arrows: crystal (remnants), m: mitochondrion, pg: plastoglobules, w: cell wall, #: internal membranes
As shown in Figure 2B and C, these plastoglobules were different in size and frequently contained an electron-dense surface area, potentially representing a lipoprotein layer (184). Based upon these observations, the cis-lycopene of our tangerine tomato variety is deposited in a lipid-dissolved physical state. This finding is consistent with previous work on Golden Jubilee tomatoes, a variety reported to contain the tangerine gene and similar globular chromoplasts (108). Globular chromoplasts containing carotenoids were previously observed in comparatively lipid-rich fruits such as peach palm (Bactris gasipaes Kunth) fruits, which contain enough lipids to dissolve all present carotenoids (111). Since tangerine tomato does not contain appreciable lipid, the cis-lycopene itself might be responsible for the oily aggregate form. A similar carotenoid deposition was reported in globular structures of the microalgae Dunaliella sp., which contains extremely high concentrations (up to 10% of the dry weight) of all-trans-β-carotene and its 9-cis-isomer (185). It was proposed that the presence the 9-cis-isomer at 40% of total β-carotene might help maintaining the oily rather than a crystalline deposition form of all Dunaliella carotenoids. Supporting this hypothesis, the visual appearance of the plastoglobules in tangerine tomato was different as compared to those of red tomato chromoplasts (Figure 2), potentially indicating a compositional difference. As described below, naturally different chromoplastidal deposition forms have previously been hypothesized to substantially influence carotenoid bioavailability.

*In vitro* studies have compared the bioaccessibility of carotenoids from a variety of fruits containing chromoplasts with differing morphology. One study found significantly
higher bioaccessibility of β-carotene from mangoes (present as globular-tubular structures) compared to carrots (crystalloid structures) (183). A human clinical study conducted by Schweiggert et al. (106) compared the bioavailability of lycopene (very small crystalloid structures) and β-carotene (liquid-crystalline structures) from papaya with dose-matched tomatoes (large crystals) and carrots (large crystals) respectively. These authors found lycopene to be 2.6 times and β-carotene 3 times more bioavailable from papaya compared to carrot, suggesting that physical deposition form may be a determinant factor in post-prandial absorption differences. Similarly, the highly different deposition forms of lycopene in tangerine and red tomatoes might help explaining the extreme differences in bioavailability noted in this study.

3.3.3 Lycopene bioavailability from tomato juices

The 10 mg lycopene dose resulted in a mean AUC value (± SEM, n=11) for total lycopene of 690.9 ± 117.8 nmol·hr/L TRL from tangerine tomato juice and 81.6 ± 32.1 nmol·hr/L TRL from red tomato juice, with significantly higher bioavailability from tangerine tomato juice (P<0.001) (Figure 3). Lycopene from tangerine tomatoes was, on average, approximately 8.5 times more bioavailable compared to red tomatoes. A HPLC-MS/MS chromatogram showing carotenoids present in a 6 hr TRL after consumption of tangerine tomato juice is shown in Figure 4.
Figure 3.3 - Post-prandial absorption of total lycopene (cis + trans) from tangerine (▲) and red (■) tomato juices. The 10 mg dose of lycopene resulted in TRL concentrations (average ± SEM) of 690.9 ± 117.8 nmol·hr/L from tangerine tomato juice and 81.6 ± 32.1 nmol·hr/L from red tomato juice, making lycopene from tangerine tomatoes, on average, 8.5 times more bioavailable than lycopene from red tomatoes.
Figure 3.4 - HPLC-MS/MS chromatogram of a 6 hr TRL after the consumption of tangerine tomato juice. HPLC-DAD-MS/MS parameters and peak labeled are given in Table 2.
One previous report from our group found a lycopene AUC of 870.2 ± 186.9 nmol·h/L TRL and C\text{max} of 189.8 ± 44.2 nmol/L TRL from tangerine tomatoes when given 13 mg with 15 g of lipid (186). These data are in good agreement with the results found in this work, although Unlu et al., did not directly compare lycopene bioavailability from tangerine tomatoes to an equal dose from red tomatoes. The profile of lycopene isomers in TRLs after consumption of tangerine tomato juice was consistent with the profile in the juice.

The average (± SEM) fractional absorption of this 10 mg dose was 47.7 ± 8.81% from tangerine tomato and 4.98 ± 1.92% from red tomato. Fractional absorption ranged from 4.59-88.3% from tangerine tomato juice and 0.24-19.0% from red tomato juice, demonstrating heterogeneity in carotenoid absorption among individuals.

Since this study was conducted using a cross-over design, each subject can be compared to themselves in terms of lycopene bioavailability from the two tomato sources. Thus, the ratio of AUC\text{tangerine}/AUC\text{red} reflects how each individual absorbed lycopene from tangerine tomatoes with reference to red. The mean AUC\text{tangerine}/AUC\text{red} (± SEM) was 52.5 ± 22.0. Only one subject absorbed less lycopene from tangerine tomato juice (AUC\text{tangerine}/AUC\text{red} = 0.51). The remaining ten of the eleven subjects absorbed between 2.3 and 247 times more lycopene from tangerine compared to red tomato juice. There was also greater variation in absorption from the red tomato juice (80 fold difference between the two most extreme subjects) than from the tangerine tomato juice (20 fold...
difference between the two most extreme subjects). The data appears heterogeneous, with some subjects gaining considerably larger fold increases in blood lycopene from tangerine tomato relative to red tomato. Specifically, subjects who have the lowest lycopene AUC_{red} on red tomato juice have the largest fold increase (lycopene AUC_{tangerine}/AUC_{red}) when consuming tangerine tomato juice and vice versa (Figure 5).
Figure 3.5 - Inter-individual differences in absorption of lycopene from red tomato juice and fold increase in lycopene absorption from *tangerine* compared to red tomato juice.
We hypothesize that this relationship between fold change and red tomato lycopene absorption is largely explained by inter-individual differences in ability to dissolve crystalline lycopene from red tomato, as subjects were more similar in their absorption of tetra-cis-lycopene from *tangerine* tomatoes. This explains the decrease in variation after consumption, and increase in absorption of lycopene from *tangerine* tomato juice, as these carotenoids are already present in lipid dissolved droplets.

Often, when discussing absorption of carotenoids, the process is considered in two phases we can loosely categorize as bioaccessibility and absorption. The bioaccessibility portion measures the amount of carotenoid that can be liberated from a food matrix and subsequently incorporated into mixed micelles, providing the theoretical maximum available for absorption. In reality, less carotenoid is absorbed than this theoretical amount, and the true amount absorbed by enterocytes and secreted into circulation is termed bioavailability. Since *tangerine* tomatoes have *cis*-lycopene present in plastoglobules and *cis*-carotenoids (including tetra-*cis*-lycopene) do not crystallize, this form of lycopene should be more bioaccessible since it does not need to be dissolved before absorption. We can thus hypothesize that the increase in lycopene AUC from *tangerine* tomatoes is a function of more dissolved carotenoid available for absorption.

Many factors introducing heterogeneity into carotenoid absorption were controlled for in this study. Our subjects were of similar age, were non-smokers, were not affected by malabsorption disorders, received the same amount of lycopene in each test meal,
received carotenoids in the same food matrix and consumed meals with the same composition. However, we still observed heterogeneity in carotenoid absorption among individuals. Other host factors, including the genetics of each individuals, which have been shown to influence provitamin A carotenoid absorption and metabolism, may be partially responsible for this observation. Leung et al. found that 2 nonsynonymous SNPs within the coding region of β-carotene oxygenase 1 (BCO1) caused a reduced catalytic activity of this cleavage enzyme in vitro as well as a reduced ability to convert β-carotene to retinyl palmitate in humans, causing higher baseline levels of β-carotene in plasma (187). The same group found that there were large variation in these affected alleles by ethnicity, suggesting this may also be a factor in carotenoid absorption and conversion (188). In vitro data has found that, contrary to conventional thought, BCO1 can act on lycopene despite the absence of a unsubstituted β-ionone ring (189). BCO1 SNPs were also recently related to a strong or weak plasma response after the consumption of watermelon or tomato juice (190), further suggesting that BCO1 plays some role in lycopene metabolism. Recent work has found that 73% of the variability of postprandial lutein in chylomicrons can be explained by SNPs in 15 genes involved in carotenoid and chylomicron metabolism (97).

Additionally, we noticed differences in total lycopene AUC values between males and females. In this study, all males had higher AUC values for lycopene on tangerine tomatoes than all females, a statistically significant increase (P = 0.004). Most studies in the literature report higher free-living serum carotenoid levels in women [43, 44], likely a
function of higher consumption of carotenoids in the diet. Additionally, it has been shown that chronic supplementation of β-carotene over one year leads to a relatively higher increase in plasma β-carotene in women (102). It has been suggested that for retinol, women have a higher transfer coefficient from the plasma to the extravascular pool, and this faster clearance is responsible for an apparent lower appearance of $^{13}$C labeled retinyl palmitate derived from $^{13}$C labeled β-carotene or retinyl acetate (103). Despite lycopene being a non-provitamin A carotenoid, this may explain an apparent lower AUC for lycopene in females in this study despite trends in the literature.

Many post-prandial studies investigating lycopene absorption use relatively high doses, above 20 mg (89). Two studies feeding 10, 30, 60, 90 and 120 mg of lycopene were given in a tomato beverage, and found no significant increases in fractional or net absorption between the 10 mg dose and the higher doses [48, 49]. This observation suggests lycopene absorption is saturable, or at minimum, reduced, at doses above 10 mg. We hypothesize that the barrier to lycopene absorption occurs more at dissolution compared to uptake. This observed saturation rationalizes the need for dose matching, as to appropriately compare bioavailability of lycopene from different food sources.

3.3.4 Phytoene, phytofluene, ζ-carotene and neurosporene bioavailability from tomato juices

The unique carotenoid profile of tangerine tomatoes allowed us to investigate the post-prandial absorption of phytoene, phytofluene, ζ-carotene and neurosporene into
chylomicrons in humans. Mean (± SEM) AUC and $C_{\text{max}}$ of these carotenoids from *tangerine* tomato juice can be found in Table 4. These lycopene precursors appear to follow approximately the same absorption curve and time-course as lycopene and other carotenoids (Figure 6). Since concentrations of phytoene, phytofluene, ζ-carotene and neurosporene were not dose matched between the two juices, we are unable to make comparisons on absorption. Some hypothesize that phytoene and phytofluene contribute to the benefits observed in those consuming tomatoes over purified lycopene (192). Additionally, phytoene and phytofluene are thought to exist primarily in *cis* configurations in plants (33), and have been shown to be more bioavailable than lycopene from red tomatoes in animals (193,194). In this study, the net amount of phytoene and phytofluene absorbed from red tomato juice was quite consistent among subjects, despite great variability in lycopene absorption. This suggests that for phytoene and phytofluene as well, physical structure plays a role in bioavailability. Tomatoes are likely the best source of phytoene and phytofluene in a Western diet [16, 51, 52] and further work is needed to elucidate the role they may play in chronic disease prevention.
Table 3.4 - Carotenoid $C_{\text{max}}$ and $\text{AUC}_{0-12\,\text{hr}}$ (mean ± SEM) baseline corrected in TRLs ($n = 11$).

<table>
<thead>
<tr>
<th>Carotenoid</th>
<th>$Tangerine$ tomato juice</th>
<th></th>
<th></th>
<th>$Red$ tomato juice</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$C_{\text{max}}$ (nmol/L)</td>
<td>$\text{AUC}_{0-12,\text{hr}}$ (nmol·h/L)</td>
<td>$C_{\text{max}}$ (nmol/L)</td>
<td>$\text{AUC}_{0-12,\text{hr}}$ (nmol·h/L)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phytoene</td>
<td>237.7 ± 37.5</td>
<td>1128.8 ± 197.7</td>
<td>28.5 ± 5.6</td>
<td>97.1 ± 14.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phytofluene</td>
<td>54.6 ± 9.6</td>
<td>317.7 ± 64.8</td>
<td>21.2 ± 3.6</td>
<td>87.3 ± 19.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$\zeta$-carotene</td>
<td>129.0 ± 23.2</td>
<td>704.9 ± 133.5</td>
<td>nd</td>
<td>nd</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Neurosporene</td>
<td>83.1 ± 15.7</td>
<td>417.7 ± 80.4</td>
<td>nd</td>
<td>nd</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tetra-cis-lycopene</td>
<td>50.3 ± 7.1</td>
<td>291.6 ±45.4</td>
<td>nd</td>
<td>nd</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cis-lycopene</td>
<td>133.5 ± 21.8</td>
<td>636.8 ± 108.5</td>
<td>13.6 ± 3.8</td>
<td>52.5 ± 31.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>All-trans-lycopene</td>
<td>14.4 ± 3.1</td>
<td>54.1 ±15.3</td>
<td>7.4 ± 2.9</td>
<td>29.1 ± 17.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total lycopene</td>
<td>144.8 ± 23.4</td>
<td>690.9 ± 117.8</td>
<td>20.5 ± 6.5</td>
<td>81.6 ± 32.1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

nd: not detected
Figure 3.6 - Post-prandial absorption of 44.9 mg phytoene (A), 13.9 mg phytofluene (B), 29.3 mg ζ-carotene (C) and 6.26 mg neurosporene (D) which are found in substantial concentrations in *tangerine* tomato juice.
3.3.5 Effects of tomato on inflammatory markers

To explore the effects of dietary intervention with tomato on biomarkers of inflammation, we assessed the gene expression profile of well-characterized cytokines and chemokines in RNA isolated from PBMCs of study subjects. These data indicated no significant effect of a single tomato containing meal on the gene expression of several pro-inflammatory transcripts, including IL-1β, IL-6, IL-10, IFN-γ and tumor growth factor β1 (TGF-β1) within PBMCs (Figure 7). Burton-Freeman et al. observed that consuming tomato paste (ca. 27 mg lycopene) in conjunction with a high fat meal, in comparison to a high fat meal alone, could significantly reduce levels of IL-6 at 360 minutes after consumption (66). A high fat meal can act as a stressor to induce oxidative stress (196). Consumption of a high fat meal has been shown to decrease flow-mediate vasodilation, more so than a low fat meal, and this stress can be mediated by the co-consumption of vitamins or antioxidants [54, 55]. Given the fact that all patients enrolled in this study were healthy subjects with no underlying inflammatory conditions or triggers, and were not challenged to promote an inflammatory state, these findings were not surprising. These results do, however; illustrate a feasible method for monitoring immunologic changes at the transcript level in future studies in other patient populations with a higher baseline level of inflammatory biomarkers.
Figure 3.7 - Ratio of transcript copies for selected cytokines IFN\(\gamma\), IL-10, IL-1\(\beta\), IL-6 and TGF-\(\beta\)1 at hour 6 divided by hour 0. Error bars represent standard deviations \((n = 10)\).
3.4 Conclusions

We investigated the post-prandial absorption of lycopene from tangerine and red tomatoes in humans. In this study, lycopene from tangerine tomatoes was, on average, 8.5 times more bioavailable as compared lycopene from red tomato juice ($P<0.001$). Within an individual, the average increase in lycopene AUC from tangerine tomatoes was 52 fold. We attribute this increase in bioavailability to tangerine tomatoes being rich in cis-lycopene and this lycopene present in lipid-dissolved globular structures in chromoplasts. In contrast, lycopene in red tomatoes, present as all-trans-lycopene, exists in large crystalline aggregates contributing to poor solubilization and comparatively lower bioavailability. Additionally, we did not observe any differences in gene transcript levels encoding inflammatory markers or cytokines after the consumption of a single tomato rich meal.

Tangerine tomatoes are a unique hybrid with the ability to greatly increase plasma lycopene and could represent a unique source of lycopene for studies of chronic disease prevention. If increased plasma lycopene is responsible for a decreased risk for certain chronic diseases, the tangerine tomato is a novel, highly bioavailable source of lycopene allowing individuals to consume reasonable amounts of tomatoes while still conferring health benefits.
CHAPTER 4 - TOMATO CAROTENOIDS PROTECT AGAINST UV-INDUCED CUTANEOUS DAMAGE AND TUMOR DEVELOPMENT IN SKH1 HAIRLESS MICE

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4.0 Abstract

Sun exposure is a major risk factor in skin cancer development. Previous work suggests tomato consumption for more than 10 weeks can mitigate ultraviolet (UV) light induced sunburn. Dietary carotenoids distributed to the skin may act as photoprotectants, free radical quenchers and antioxidants to protect skin against UV-induced damage. In addition to lycopene, tomatoes have higher levels of UV-absorbing carotenoids (phytoene/phytofluene) compared to other fruits and vegetables. We hypothesize that by decreasing the UV-induced skin inflammatory response tomato consumption would ultimately protect against skin cancer. Male and female SKH1 hairless and immunocompetent mice \((n=180)\) were fed AIN-93G or AIN-93G + 10% tangerine or red tomato powder for 35 weeks. From weeks 11-20, mice \((n=120)\) were exposed to 2240 J/m\(^2\) UVB light, 3 times per week and resulting tumors were tracked weekly. Control mice were fed the diets but not exposed to UV. Mice consuming tomato diets developed significantly fewer tumors compared to mice on control diets \((P<0.001)\). The effects of tomato supplementation and UV exposure on carotenoids in plasma and skin and tumor statistics/grade were determined. These findings provide impetus for continued investigation of tomato carotenoids on human skin outcomes.
4.1 Introduction

One of the principle functions of carotenoids in plants is to act as photoprotectants (18). Carotenoids help to funnel energy away from chlorophyll and the photosynthetic apparatus and can scavenge singlet oxygen (199,200). It is reasonable to think that carotenoids may play a similarly protective role in humans after consumption of carotenoid rich fruits and vegetables. Lycopene has been shown to be the most effective singlet oxygen quencher of the carotenoids (201). Carotenoids are deposited in the skin of humans (172,202) where they are, in theory, present and able to protect from ultraviolet damage. The sun emits ultraviolet (UV) radiation from approximately 100-400 nm, which can be further divided into UVA (315-400 nm), UVB (250-315 nm and UVC (~100-280 nm) (112). UVB light appears to be much more effective in producing cancer in animals compared to UVA light, while most UVC light is filtered out by the atmosphere (113). Damage from UV radiation can cause reactive oxygen species when light of a suitable wavelength interacts with a chromophore. Photooxidative damage can affect lipids proteins and DNA and is involved in skin aging, erythema, photodermatoses and skin cancer (114). An individual’s sensitivity to UV radiation can be assessed by determining a minimal erythemic dose (MED), or the dose needed to cause reddening of the skin 24 hours after exposure to UVB radiation (115). MED levels can differ dramatically between individuals and are related to skin type, categorized using the Fitzpatrick scale from type I to type IV. Skin type I denotes people with white or freckled skin, light blue or green eyes, light blonde or red hair with a high sensitivity to sunburns while skin type IV denotes people with black skin, dark eyes and black hair,
almost never suffering from sunburns. Melanin levels play a large role in an individual’s susceptibility to UV-induced damage, as melanins scatter and absorb UV light (116).

It is hypothesized that carotenoids act as photoprotectants (117), free radical quenchers (55) and antioxidants (118), all of which may play a role in protecting the skin against UV-induced damage. Studies in mice that are both hairless and immunocompetent (SKH1) have shown that β-carotene and canthaxanthin can be protective against UVB induced damage (119–122). Some evidence from human clinical trials suggest that lycopene and tomato paste can decrease erythema resulting from UV light exposure (123). Sies and Stahl have conducted a number of experiments that suggest tomato-derived carotenoids can protect against UV-induced erythema. They have conducted five studies in Fitzpatrick type II skin individuals using various carotenoid diets (feeding tomato paste, carrot juice, a lycopene supplement, a lycopene drink and synthetic lycopene) for 10-12 weeks. In the tomato paste study, subjects consumed 40 g of tomato paste with 10 g olive oil providing 16 mg of lycopene per day (123). In the carrot juice study, subjects consumed 400 mL of a unique carrot juice providing 10 mg lycopene and 5.1 mg β-carotene (114). In the lycopene supplement study, subjects consumed a soft gel capsule with tomato extract providing 9.8 mg lycopene and 0.4 mg β-carotene per day (124). In the lycopene drink group, subjects consumed a lycopene drink derived from tomato extract, containing 8.2 mg of lycopene and 0.4 mg β-carotene per day (124). The synthetic lycopene study had subjects consume 10.2 mg of synthetic lycopene per day (124). All studies led to increases in serum carotenoids and a less substantial increase in
skin carotenoids as determined by HPLC and Raman spectroscopy respectively. After 10 or 12 weeks of supplementation, all groups except the synthetic lycopene had a significant decrease in a*-value (change in redness of skin) as compared to baseline. This suggests that other carotenoids in tomato products besides lycopene (including phytoene and phytofluene) may play a role in protection against UV-induced erythema and potential for these plant pigments to protect against skin cancer.

The objective of this study was to determine whether the dietary consumption of *tangerine* tomatoes or red tomatoes, as compared to a tomato-free diet, can differentially reduce the UVB-induced tumor promotion and progression after chronic UVB exposure in male and female SKH1 murine skin.

### 4.2 Materials and methods

#### 4.2.1 Experimental design

Four week-old male and female outbred SKH1 hairless mice (Charles River Laboratories, Wilmington, MA) were purchased and housed in a facility approved by the American Association for the Accreditation of Laboratory Animal Care and OSU’s Institutional Animal Care and Use Committee (2010A0000083). Mice were fed a control diet, a 10% *tangerine* tomato powder diet or a 10% red tomato powder diet.
Mice began on their specified diets upon receipt and remained on their diets for the duration of the 35 week long study. At the beginning of week 11 through week 20, mice on their respective diets were exposed to 1 M.E.D. UVB (2240 J/m²) dorsally using Phillips DS40UVB lamps (American Ultraviolet Company, Lebanon, IN) three times weekly on non-consecutive days. During weeks 21-35, mice continued on their respective diets, without any additional UV exposure. An experimental design schematic is simplified in Figure 4.1. This treatment protocol induces papilloma growth beginning at 6-10 weeks in males and 10-12 weeks post-UVB exposure in females, and culminates in squamous cell carcinoma development at approximately 25 weeks. Tumor number and size were measured weekly starting at week 18. Age and gender matched control animals were fed each of the diets but not exposed to UVB radiation. The number of animals was determined based on consultation with the biostatistics core at OSU and the amount of tissue necessary for completion of all of the assays.
Figure 4.1 - Experimental design to determine whether the dietary consumption of tangerine tomatoes or red tomatoes will differentially reduce the UVB-induced tumor promotion and progression after chronic UVB exposure in male and female SKH1 murine skin.
4.2.2 Tomato diets

Red and tangerine tomatoes (*Solanum lycopersicum*) were grown at the OSU North Central Agricultural Research Station in Fremont, OH. *Tangerine* varieties FG04-167, FG04-169 and 7531, and Red variety PS696, were harvested and delivered to The Ohio State University Food Industries Center, where the tomatoes were immediately diced and frozen at -40 °C. The tomatoes were then freeze dried. The dry tomato tissue was then pulverized in a vertical cutter mixer chopper and stored in vacuum sealed plastic bags in the dark at -20 °C. This dry tomato powder was then sent to Research Diets, Inc. (New Brunswick, NJ) to be incorporated at a 10% (w/w) level into a modified AIN-93G based feed and made into pellets. The control feed contained corn starch and dextrose in place of tomato powder to adjust for macronutrient composition (192). All feed was stored at -20 °C throughout the duration of the study, until it was weighed and distributed to the mice cages. The feed was replaced in the animal cages every 2-3 days to minimize carotenoid oxidation.

4.3.3 Extraction of carotenoids from murine skin and blood plasma

All extractions were performed under red light, and samples were handled quickly to prevent degradation of carotenoids during extraction. Mouse skin was extracted using a method developed specifically for these samples. Briefly, ~300 mg of frozen skin tissue was crushed using liquid nitrogen in a cell crusher. The resulting powder was weighed into glass centrifuge tubes. Water (1 mL) and ethanol with 1% butylated hydroxytoluene (BHT) (1 mL) were added to the pulverized skin and the slurry was vortexed for 15 sec.
Next, a 5:1 hexane:dichloromethane (DCM) solution (5 mL) as added and samples were probe sonicated for 8 sec and then centrifuged for 5 min at 1000 x g. The upper non-polar layer (containing carotenoid) was removed, and the samples were re-extracted with 5 mL additional hexane/DCM solution. Extracts were then pooled and evaporated to dryness under nitrogen gas. Samples were stored at -80 °C until analysis via HPLC-MS/MS which occurred within two days.

Plasma carotenoids were extracted modifying volumes for a small sample size from a previously described method (203). Briefly, 200 μL of plasma was added to 200 μL of ethanol with 0.1% BHT. The sample was vortexed for 15 sec and 2 mL of 10:6:7:7 v/v/v/v hexane:ethanol:acetone:toluene (HEAT) was added, vortexed and centrifuged for 5 min at 1000 x g. The top layer was then removed and extracted once more with HEAT and the organic layers were pooled and evaporated to dryness under nitrogen gas. All extracts were at -80 °C prior HPLC-MS/MS analysis.

4.3.4 High performance liquid chromatography tandem mass spectrometry (HPLC-MS/MS) of murine skin and plasma extracts

Extracts of skin tissue and blood plasma were separated using two separate reversed phase HPLC methods. For each, an Agilent 1260 HPLC (Santa Clara, CA) was interfaced with a QTRAP 5500 mass spectrometer (AB SCIEX, Carlsbad, CA) using an atmospheric pressure chemical ionization (APCI) probe operated in positive ion mode.
Extracts of skin tissue were separated using a Sunfire™ 4.6x150 mm, 5 μm pore size, C18 column (Waters Corp, Milford, MA) at 35 °C. Solvents A: 80:18:2 methanol:water:2% (w/v) aqueous ammonium acetate, and B: 78:20:2 MTBE:methanol:2% (w/v) aqueous ammonium acetate was used to separate the carotenoids of interest using the following linear gradient: initial conditions at 40% B to 63% B over 15 min, to 100% at 17 min and return to initial conditions for 3 min, for a total run time of 20 min. The flow rate was 1.3 mL/min. Carotenoids were monitored as a summation of multiple reaction monitoring products of $m/z$ as follows: phytoene 545>463, 421, 395, 327; phytofluene 534>461, 393, 325; ζ-carotene 541>457, 349, 271; neurosporene 539>457, 415, 389; lycopene 537>455, 269. Spectral details and mass defects can be found in Table 3.2. Dwell times were maximized to maintain 15 data points across each peak.

Extracts from murine plasma were analyzed using a C30 4.6x250 mm, 3 μm pore size column with the same method reported in chapter 3 for analysis of carotenoids in TRLs (Table 3.2).

Dorsal mouse skin was snap frozen immediately after sacrifice for carotenoid analysis. Whole blood was centrifuged to separate plasma from red blood cells, and plasma was frozen for carotenoid analysis. All samples were stored at -80 °C until extraction. Carotenoids were quantified using external calibration curves of phytoene, phytofluene, ζ-carotene and neurosporene, which were isolated using semi-preparative HPLC.
Lycopene was purified using methods that have been previously developed for external curves (164). Standard reference materials were created from *tangerine* and red tomato extracts to monitor MS response throughout the day.

4.2.5 *Measuring skin fold thickness*

Skin fold thickness, as measured using digital calipers was used to measure inflammatory response and edema 48 hours after UVB exposure (204). Forty eight hours after the first UVB exposure has been found to be the time of peak inflammation.

4.2.6 *Monitoring tumor progression*

Tumors were tracked by 2 dimensional measurement using digital calipers once per week starting at week 18 (204). Briefly, after sacrifice, tumors from each mouse receiving UVB treatment were harvested and underwent histological grading (205). Hematoxylin and eosin stained tissue were graded in a blinded way by a board-certified veterinary pathologist. Grades of hyperplasia, papilloma (grades 1-3), microinvasive squamous cell carcinoma (grades 1-3) or full invasive squamous cell carcinoma (grades 1-3) were assigned (206).
4.3 Results and discussion

4.3.1 Animal weights

All animals consumed the three diets and gained weight. Male mice were given diets to provide approximately 5 g food/day while female were given approximately 4 g/day. The average and standard deviation of the weights of the mice at week 35, prior to sacrifice are in Table 4.1. Male mice were significantly heavier than female mice ($P < 0.001$). Although there was a trend for mice on tomato diets to be heavier, there were no statistically significant differences found (Figure 4.1). Others have also found that rodents who consume tomato diets are heavier than mice on purified AIN-93G diets (207) while some have found no difference (192).
Table 4.1 - Body weights of mice at 35 weeks

<table>
<thead>
<tr>
<th>Gender</th>
<th>Diet</th>
<th>No UVB Weight (g) ± standard deviation</th>
<th>UVB Weight (g) ± standard deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female</td>
<td>Control</td>
<td>27.3 ± 2.1</td>
<td>27.1 ± 2.6</td>
</tr>
<tr>
<td></td>
<td>Tangerine</td>
<td>30.3 ± 2.4</td>
<td>29.0 ± 3.6</td>
</tr>
<tr>
<td></td>
<td>Red</td>
<td>29.5 ± 2.6</td>
<td>27.8 ± 2.1</td>
</tr>
<tr>
<td>Male</td>
<td>Control</td>
<td>36.8 ± 4.4</td>
<td>36.4 ± 3.2</td>
</tr>
<tr>
<td></td>
<td>Tangerine</td>
<td>38.4 ± 4.6</td>
<td>43.2 ± 3.8</td>
</tr>
<tr>
<td></td>
<td>Red</td>
<td>40.2 ± 2.6</td>
<td>38.1 ± 2.3</td>
</tr>
</tbody>
</table>
Figure 4.2 – Final body weights of male (A) and female (B) SKH1 mice, on control, tangerine and red tomato diets, unexposed and exposed to UV light. Open bars are not exposed to UV, closed bars are exposed to UV. No significant differences in body weights are noted within a gender between diets. Male mice are significantly heavier than female mice ($P < 0.001$).
4.3.2 Skin fold thickness

Skin fold thickness 48 hours after the first exposure to UV radiation can be used as a rough measurement of the inflammation response. Male mice had significantly thicker skin than female mice ($P < 0.001$). This held true even in mice not exposed to UV denoting that there are fundamental differences in the physiology of skin between the sexes. There was no significant differences in skin fold thickness between the control and tomato diets, but there was a numerical trend towards lower inflammation after tomato consumption (Figure 4.2). Perhaps the tomato carotenoids deposited in the skin of the mice are better able to scavenge free radicals produced during the UV exposure resulting in less inflammation in these mice. However, the outbred SKH1 have high inter-individual variability which makes it difficult to make any decisive conclusions about the effects of tomato supplementation on inflammatory response post-UV exposure.
Figure 4.3 – Mean skin fold thickness 48 hours post-first UV exposure. Open bars are not exposed to UV, closed bars are exposed to UV. Both genders had significantly thinner skin when not exposed to UV and male skin was significantly thicker than female skin ($P < 0.001$). No significant differences exist within the diet groups.
4.3.3. Carotenoid profile in tomato diets

The control, tangerine and red tomatoes were extracted and analyzed for carotenoids. Photos of the diets can be found in Figure 4.3. The control diet did not contain any carotenoids. Phytoene and phytofluene were present in approximately twice the concentration in the tangerine tomato diet compared to the red tomato diet. Total lycopene was approximately three times higher in the red tomato diet compared to the tangerine diet. About 1% of the lycopene in the tangerine tomato diet was present as all-trans-lycopene while 82% of the lycopene in the red tomato diet was present as all-trans. Overall, the tangerine tomato diet had approximately a 20% higher level of total carotenoids compared to the red diet. ζ-carotene, neurosporene and tetra-cis-lycopene were absent in the red tomato diet, and β-carotene was absent in the tangerine tomato diet. Diets were formulated based on equal weight addition of tomato powder and are therefore not matched for any carotenoid levels.
Figure 4.4 – Photographs of (A) control, (B) *tangerine* and (C) red tomato diets.
Table 4.2 – Carotenoid composition of *tangerine* and red tomato fortified diets.

<table>
<thead>
<tr>
<th>Carotenoid</th>
<th>AIN-93G + 10% <em>tangerine</em> tomato (mg/kg DW)</th>
<th>AIN-93G + 10% red tomato (mg/kg DW)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phytoene</td>
<td>64.1</td>
<td>37.0</td>
</tr>
<tr>
<td>Phytofluene</td>
<td>17.8</td>
<td>9.6</td>
</tr>
<tr>
<td>ζ-carotene</td>
<td>22.9</td>
<td>nd</td>
</tr>
<tr>
<td>Neurosporene</td>
<td>8.2</td>
<td>nd</td>
</tr>
<tr>
<td>Tetra-<em>cis</em>-lycopene</td>
<td>18.6</td>
<td>nd</td>
</tr>
<tr>
<td>Other-<em>cis</em>-lycopene</td>
<td>2.8</td>
<td>10.9</td>
</tr>
<tr>
<td>All-<em>trans</em>-lycopene</td>
<td>0.8</td>
<td>50.0</td>
</tr>
<tr>
<td>β-carotene</td>
<td>nd</td>
<td>4.6</td>
</tr>
<tr>
<td>Total lycopene</td>
<td>22.1</td>
<td>60.9</td>
</tr>
<tr>
<td>Total carotenoids</td>
<td>135.0</td>
<td>111.9</td>
</tr>
</tbody>
</table>

nd: not detected
4.3.4 Carotenoid levels in skin and blood plasma

Carotenoids were measured in murine skin and blood plasma using HPLC-MS/MS and details can be found in Table 4.3. Phytoene and phytofluene in skin were quantified using mass spectrometry while ζ-carotene and all forms of lycopene were quantified using photodiode array detection. Phytoene, phytofluene, ζ-carotene and tetra-cis-lycopene in plasma were quantified with photodiode array detection, and neurosporene, other-cis-lycopene and all-trans-lycopene were quantified using a sum of the MS/MS transitions. A chromatogram is shown in Figure 4.5 with the skin method in panel A and the plasma method in panel B.

Interestingly, there were no statistically significant differences in concentrations of lycopene in plasma or skin between the genders or within the diets. Since there was approximately 3 times less lycopene delivered in the tangerine tomato diet, this lycopene is considerably more bioavailable compared to the lycopene in the red tomato diet. This is consistent with what we have seen in human studies, with higher bioavailability of lycopene from tangerine tomatoes.

Carotenoid levels in both plasma and skin were of similar magnitude as found by Kopec et al. where 10% tangerine tomato diets were fed for 10 weeks, mice were exposed to a single dose of UVB light (1 M.E.D.) and scarified 48 hours later (207). Lycopene was the carotenoid present in highest concentration in both plasma and skin in both tomato diets, despite not being the most prevalent carotenoid in the tangerine tomato diet.
Despite 3 times higher concentrations of phytoene in the tangerine tomato diet, lycopene is present at 10 fold higher levels in skin, and 2–4 fold higher concentrations in plasma. This is contrary to what has been found by other groups that have found that phytoene is more bioavailable than lycopene (193,194).
Table 4.3 – Concentration of carotenoids in murine skin fed diets containing *tangerine* and red tomato powders.

<table>
<thead>
<tr>
<th>Carotenoid</th>
<th>Fed <em>tangerine</em> tomato diet pmol/g skin (std. dev.)</th>
<th>Fed red tomato diet pmol/g skin (std. dev.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Male No UV UV Male No UV UV Male No UV UV Female No UV UV Female No UV UV</td>
<td></td>
</tr>
<tr>
<td>Phytoene</td>
<td>57.0 (20.5) 63.6 (30.0) 111.0 (56.2) 108.1 (31.8) 33.9 (17.3) 77.0 (47.2) 123.4 (34.1) 56.8 (26.5)</td>
<td></td>
</tr>
<tr>
<td>Phytofluene</td>
<td>19.8 (4.0) 17.7 (6.3) 30.6 (11.6) 21.8 (7.9) nq nq nq nq</td>
<td></td>
</tr>
<tr>
<td>ζ-carotene</td>
<td>185.2 (76.1) 362.1 (272.1) 998.5 (1108.6) 294.2 (111.3) nd nd nd nd</td>
<td></td>
</tr>
<tr>
<td>Tetra-*cis-*lycopene</td>
<td>120.6 (64.4) 162.5 (81.4) 207.0 (106.2) 146.8 (40.7) nd nd nd nd</td>
<td></td>
</tr>
<tr>
<td>All-<em>trans</em> + other-*cis-*lycopene</td>
<td>424.8 (178.6) 882.4 (708.9) 1772.9 (1708.3) 672.7 (266.1) 235.5 (261.6) 307.5 (123.6) 734.2 (599.1) 1081.7 (796.5)</td>
<td></td>
</tr>
<tr>
<td>Total lycopene</td>
<td>545.4 (241.7) 1044.8 (783.1) 966.5 (424.5) 881.2 (309.8) 235.5 (261.6) 307.5 (123.6) 734.2 (599.1) 1081.7 (796.5)</td>
<td></td>
</tr>
</tbody>
</table>

nq: not quantifiable; nd: not detected
Table 4.4 – Concentration of carotenoids in plasma of mice consuming *tangerine* and red tomato diets.

<table>
<thead>
<tr>
<th>Carotenoid</th>
<th>Fed <em>tangerine</em> tomato diet</th>
<th>Fed red tomato diet</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Male</td>
<td>Female</td>
</tr>
<tr>
<td></td>
<td>No UV $n = 9$</td>
<td>UV $n = 10$</td>
</tr>
<tr>
<td>Phytoene</td>
<td>12.1 (4.0)</td>
<td>62.0 (29.1)</td>
</tr>
<tr>
<td>Phytofluene</td>
<td>10.4 (3.3)</td>
<td>21.6 (9.8)</td>
</tr>
<tr>
<td>ζ-carotene</td>
<td>20.0 (8.4)</td>
<td>44.4 (17.4)</td>
</tr>
<tr>
<td>Neurosporene</td>
<td>15.7 (6.9)</td>
<td>32.4 (12.9)</td>
</tr>
<tr>
<td>Tetra-cis-lycopene</td>
<td>26.2 (17.5)</td>
<td>35.2 (21.7)</td>
</tr>
<tr>
<td>Other-cis-lycopene</td>
<td>20.4 (8.4)</td>
<td>38.3 (16.1)</td>
</tr>
<tr>
<td>All-trans-lycopene</td>
<td>5.9 (2.2)</td>
<td>13.3 (4.2)</td>
</tr>
<tr>
<td>5-cis-lycopene</td>
<td>5.8 (2.7)</td>
<td>11.2 (3.5)</td>
</tr>
<tr>
<td>Total lycopene</td>
<td>57.3 (30.1)</td>
<td>99.6 (44.1)</td>
</tr>
</tbody>
</table>

All values are in nmol/L plasma (std. dev.).
Figure 4.5 – HPLC-MS/MS chromatograms used to separate carotenoids in murine skin (A) and plasma (B).
Trends in carotenoid levels between animals exposed to and not exposed to UV are inconsistent between analytes in this study. Others have found that UV radiation decreases carotenoids in skin (but not plasma) when skin is collected 48 hours after a one-time exposure (207). A similar result has been observed in humans, where reductions in plasma carotenoids were noted after individuals were exposed to UVA and UVB light over a two week period (208). The possibility exists that our skin samples were taken too long after the last UV exposure (15 weeks) and by this point, UV differences in carotenoid content in the skin were lost. The high variability noted with the SKH1 outbred mouse creates challenges for conducting statistical analyses. Large heterogeneity is consistent with human populations but requires large numbers of animals for studies to be powered to see differences between treatments. In addition, the SKH1 mouse is a well-accepted model for non-melanoma skin cancer, but not necessarily a good model for studying carotenoid uptake and deposition into tissues. Rodents tend to not be very good models for carotenoid absorption and distribution since they do not absorb carotenoid intact unless they are fed at supra-physiological doses (209). In this study, the dose of tomato given to the mice was chosen to be sufficient to produce plasma lycopene levels that are consistent with humans consuming a diet rich in lycopene containing foods. Despite a diet containing 10% by weight tomato powder, mice reached 50-70 nmol/L lycopene. Studies in free-living humans have shown a range of lycopene in plasma, from 200-1000 nmol/L (210,211). This again provides additional evidence that carotenoid bioavailability and metabolism differs between mice and humans.
Hata et al. found an average of 69 ng lycopene (129 pmol lycopene/g) and 65 ng phytoene (120 pmol phytoene/g) per gram skin, in the range of what we see in the SKH1 mice. Ribaya-Mercado found approximately 1.5 nmol lycopene/g skin as determined using HPLC while Mayne et al. found from 100-800 ng carotenoids/g skin (approximately 186-1493 pmol carotenoid/g skin) as determined using Raman resonance spectroscopy (212,213). This data suggests that SKH1 mice accumulate carotenoids in skin in reasonably similar concentrations as do humans.

Lycopene has been shown in cell culture to follow a U-shaped curve in terms of its benefit in fibroblasts exposed to UVB light. At optimal levels (0.05 nmol/mg protein), lycopene was shown to decrease UV-induced formation of thiobarbaturic acid-reactive substances (TBARS) to 40-50% the levels of controls, indicating protection against lipid peroxidation (214). A study in hairless mice found that supplementation of diets with palm fruit carotenoids can decrease TBARS from controls (215).

4.3.5 Tumor incidence, progression and grade
To our knowledge, this is the first study investigating the effects of tomato consumption on non-melanoma skin cancer. Tumor number and progression from 20 to 35 weeks can be seen in Figure 4.6. Male mice on control diets got significantly more tumors than male mice on red tomato diets ($P = 0.0146$). Male mice on control diets got significantly more tumors than mice pooled on tomato diets ($P = 0.017$). Male mice on control diets were not significantly different from male mice on tangerine diets ($P = 0.2197$). There are no
significant differences in tumor number for any of the female mice. The average number of tumors per female control mouse is only approximately 1. This suggests that the period of UV exposure was too short to induce significant tumor development. The presence of differences in males and the absence of an effect in female may be a function of sex differences in the development on non-melanoma skin cancer, which have been shown previously (205). Or, it is possible that not enough tumors were induced in the females to be able to truly detect differences, given that they exist.

No significant differences were noted between the tomato diets within a gender when looking at tumor burden, a measure of the average area of tumor (mm$^2$) per mouse. Additionally, carotenoids are deposited in the fat depots of these mice, as demonstrated in Figure 4.7 by the pigmentation of the inguinal fat of these animals.
Figure 4.6 – Tumor number progression in male (A) and female (B) mice.
Figure 4.7 – Color of adipose reflects dietary exposure to carotenoids.
Tumors were graded by a board certified veterinary pathologist. Figure 4.8 shows the number of tumors per mouse, breaking down into the number of benign and malignant tumors per mouse. Male mice had numerically fewer total tumors, benign tumors and malignant tumors when on tomato diets compared to control diets. Female mice on red tomato diets had numerically fewer tumors than female mice on control or tangerine diets.
Figure 4.8 – Total, benign and malignant tumors on male and female UV exposed mice on control, \textit{tangerine} and red tomato diets.
4.4 Conclusions

Overall, male mice who consumed tomato containing diets developed fewer squamous cell carcinomas as the result of exposure to UVB light compared to male mice who did not consume tomatoes. Highly sensitive HPLC-PDA-MS/MS methods were developed to identify and quantify carotenoids in murine plasma and skin. SKH1 male and female mice consuming a tangerine or red tomato powder containing diet mice accumulate similar levels of lycopene in their plasma and skin despite there being about 3 times less lycopene in the tangerine tomato diet. This further confirms an increase in bioavailability of lycopene from tangerine tomatoes. Additionally, the concentrations of carotenoids in the skin of humans are similar to the concentrations determined in these tomato fed SKH1 mice. These data suggest that further research should investigate the role that tomatoes and tomato phytochemicals plan in the mediation of non-melanoma skin cancers.
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