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PHYSICAL AND CHEMICAL STABILITY OF ALL-TRANS LYCOPENE AND OTHER TOMATO CAROTENOIDS IN VITRO

DISSERTATION

Submitted in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

in the Graduate School of The Ohio State University

by

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The Ohio State University
1999

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Food Science & Nutrition Graduate Program
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Minhthy Le Nguyen

1999
ABSTRACT

During the past few years, the tomato carotenoid, lycopene, has become increasingly recognized as an important dietary phytochemical with unique value in human health. The accumulating epidemiological evidence supporting the association between diets rich in the tomato products, therefore lycopene, and certain health benefits are very compelling, leading to an increasing interest the stability and bioavailability of this phytochemical in the diet. Meanwhile, the consumption of tomatoes and related products in the United States are on the rise. Furthermore, the ratio of lycopene in the all-trans geometrical forms versus those in the cis configuration from fresh tomatoes has been observed to be significantly different than those from human blood and tissues. This difference, specifically, has prompted considerable attention to the formation and potential biological significance of lycopene isomers, especially in terms of possible unique or altered physiological roles associated with a particular geometrical configuration.

Some investigators have suggested that lycopene undergoes isomerization reactions during thermal processing of tomato products and the level of newly formed species in the cis configurations are reflected in the blood and tissues following absorption and distribution. A definite account of lycopene isomerization processes and end points requires extensive experimental data which are not currently available. The
objectives of this work are to monitor the stability of lycopene and other tomato
carotenoids towards isomerization during typical food processing using various tomato
varieties and secondly, to determine if isomerization reactions take place during the
digestive process.

First, the thermal stability during food processing of lycopene in the all-trans
geometrical configuration was evaluated using industrial processing facilities and
conditions. The results indicated that, lycopene, in contrast to β-carotene, was stable
against isomerization reactions during thermal processing and following an 18-month
storage period, independent of the production variables such as product type, container
type, moisture content, tomato variety and severity of heat treatments. Second, when
tomato mutant varieties with distinct differences in the distribution of chromoplastid
carotenoid pigments, including β-carotene, δ-carotene, γ-carotene, lutein and
prolycopene-a poly-cis lycopene isomer, were heat-treated in either distilled water or a
water/oil mixture, lycopene, δ-carotene and γ-carotene were also found to remain stable in
the all-trans configurations. β-carotene and lutein, on the other hand, readily isomerized
to the cis geometrical forms as a result of both thermal treatments, suggesting either an
isomerization mechanism involving either localized specificity within tomato cells or
structural selectivity among tomato carotenoids. Third, no isomerization of tomato
carotenoids including lycopene, occurred either during the gastric or intestinal phases of
digestion as monitored by an in vitro digestion protocol.

Additionally, high levels of lycopene cis-isomers were observed in Caco-2
human colon cells, which have been incubated with micellar lycopene derived from the
in vitro digestion protocol. Chromatographic analysis of biological samples such as
human blood chylomicron fractions from individuals consuming lycopene-containing products, human serum and prostate tissues, likewise, yielded high cis to trans isomeric ratios. These findings help to confirm that the high level of various lycopene cis isomers found in biological fluids and tissue are not formed during food processing, food storage or during digestion, and therefore most likely arise after absorption. Ultimately, these findings not only provide a better understanding on the impact of food processing and operational variables on lycopene's stability in vitro but they also contribute toward the overall effort to assess nutritional and physiological implications of this phytochemical in the diet. The implications of this knowledge will have an impact on basic lycopene research as well as in the design of lycopene intervention trials in humans.
Dedicated with affection to my mother, Mrs. Le Thi Ba,

for the gift of life and her inspiring examples of

devotion, perseverance, generosity and selflessness.
ACKNOWLEDGEMENTS

I am grateful to the Lord, our God, for making this endeavor both possible and successful by granting me the clarity of mind and strength of body over the past five years. I am thankful for his infinite wisdom, placing me in the path of supportive and caring individuals to help me reach my goals.

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conduct *in vitro* studies in Greensboro, Dr. Zora Djuric (The Barbara Ann Karmanos Cancer Institute, Wayne State University, MI) for providing the blood samples and Dr. Joyce Swanson for the generous accommodation at Cornell University during the chylomicron isolation trials.

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Finally, I would like to thank my Mom and Dad, my wife and her family, my aunt and her family for their faith, encouragement and unwavering support. As for Noah, thank you for being the source of strength and peace even at your young tender age.

To all these individuals whom I am thankful to have known, learned from and worked with, I offer my sincerest prayer for their health.
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FIELDS OF STUDY

Major Field: Food Chemistry
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<tr>
<td>ATCC</td>
<td>American type culture collection</td>
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<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BEA</td>
<td>Lycopene beadlets</td>
</tr>
<tr>
<td>BET</td>
<td>High β-carotene tomatoes</td>
</tr>
<tr>
<td>BHT</td>
<td>Butylated Hydroxyltoluene</td>
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<td>Caco-2 cells</td>
<td>Cell line derived from human adenocarcinoma of the colon</td>
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<td>CIN</td>
<td>Cervical Intraepithelia Neoplasia</td>
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<td>DEL</td>
<td>High δ-carotene tomatoes</td>
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<td>DMEM</td>
<td>Dulbecco’s minimum essential medium</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic Acid</td>
</tr>
<tr>
<td>E</td>
<td>The all-trans form of a carotenoid</td>
</tr>
<tr>
<td>FCS</td>
<td>Fetal calf serum</td>
</tr>
<tr>
<td>FIB</td>
<td>Lycopene microfiber</td>
</tr>
<tr>
<td>GGPP</td>
<td>Geranylgeranyl pyrophosphate</td>
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<tr>
<td>GUA</td>
<td>Concentrated guava samples</td>
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<td>HBSS</td>
<td>Hank’s balanced salts solution</td>
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<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<tr>
<td>HEPES</td>
<td>N-2-Hydroxyethylpiperazine-N-2′-ethanesulfonic acid</td>
</tr>
<tr>
<td>HDL</td>
<td>High-density lipoproteins</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
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<tr>
<td>IPP</td>
<td>Isopentenyl pyrophosphate</td>
</tr>
<tr>
<td>IUB</td>
<td>International Union of Biochemists</td>
</tr>
<tr>
<td>IUPAC</td>
<td>International Union of Pure and Applied Chemistry</td>
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<tr>
<td>λ&lt;sub&gt;max&lt;/sub&gt;</td>
<td>Maximum absorbance peak of the ultra-violet spectrum</td>
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<td>LDL</td>
<td>Low-density lipoproteins</td>
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<td>LOW</td>
<td>Yellow tomatoes with low carotenoid content</td>
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<td>LYC</td>
<td>High lycopene tomatoes</td>
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<td>TGFalpha</td>
<td>Transforming growth factor alpha</td>
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<tr>
<td>MVA</td>
<td>Mevalonic acid</td>
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<tr>
<td>NMR</td>
<td>Nuclear magnetic resonance</td>
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<td>PIL</td>
<td>Lycopene pills</td>
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<td>Tangerine-type tomatoes containing prolycopene</td>
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<td>RED</td>
<td>Crimson-type tomatoes</td>
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<td>ROS</td>
<td>Rosehip puree samples</td>
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<td>UV</td>
<td>Ultra-violet electromagnetic radiation</td>
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INTRODUCTION

The tomato carotenoid, lycopene, has attracted considerable attention recently as epidemiological evidence continues to suggest that it may provide protection against cancer and other degenerative diseases influenced by radical reactions (Gey, 1993; Bendich, 1989; Giovannucci et al., 1995). For example, a recent comprehensive review of 72 independent epidemiological studies revealed that intake of tomatoes and its products along with blood lycopene level are inversely associated with the risk of developing cancers at several anatomic sites including the prostate gland, stomach and lung. Data were also suggestive for breast, cervical, colorectal, esophageal, oral cavity, and pancreatic cancers (Giovannucci, 1999). These observational findings are substantiated by laboratory data from human cell-culture and animal studies, showing lycopene to be very effective in trapping free radicals and quenching singlet oxygen - protecting oxidizable substrates from harmful degradative reactions (Kim, 1995; Matsushima-Nishiwaki et al., 1995; Nagasawa et al., 1995; Stahl and Sies, 1993; Conn et al., 1991; Countryman et al., 1991; Wang et al., 1989).

Lycopene, as the predominant carotenoids in human plasma and a variety of anatomic sites, has been shown to exist in several geometrical configurations, where the cis isomers content ranges from 50-88% of the total lycopene level (Krinsky, 1990; Schmitz et al., 1991; Stahl et al., 1992, 1992, 1993; Emenhiser et al., 1995; Clinton et
The presence of *cis* lycopene isomers in biological samples at elevated levels and in higher numbers compared to fresh tomato suggests an isomerization mechanism involving either food processing or *in vivo* conditions. Another possibility is that lycopene in the *cis* configuration is better absorbed or preferentially deposited under physiological conditions than its all-trans counterpart. The existing hypothesis assumes that the high percentage of lycopene *cis* isomers in human biological samples is due in part to consumption of heat treated tomato products containing *cis* isomers of lycopene (Sies and Stahl, 1982; Schierle et al., 1997; van het Hof et al., 1998). Nonetheless, the combination of accumulating evidence of lycopene’s health implications and the markedly different isomer profile of *cis* lycopene isomers in humans compared to lycopene from fresh tomatoes has prompted considerable interest in understanding the role of lycopene in the diet, its stability in foods and the significance of isomer formation in the overall mechanism of lycopene’s biological activities.

Despite the growing interest in lycopene's role in human nutrition and health and the proliferation of research studies involving lycopene, there are in fact many important questions regarding the fundamental properties of lycopene which remain unanswered. Among these questions concerning the nature of lycopene in the context of human health, a number of relevant issues include: What is the physical state of lycopene in the various food and tissue matrices? How will the chemistry of the molecule vary with time and environmental factors as lycopene undergoes food processing and digestion? Why is the ratio of *cis-trans* isomers *in vivo* different compared to that of fresh tomatoes? At which point from biosynthesis to absorption
and distribution does the isomer distribution begin to shift towards a higher percentage of cis-isomers? The answers to these questions are critical to the overall process of elucidating lycopene's role as an important dietary phytochemical in human nutrition and health.

In order to provide answers to some of the questions above, the overall objective of this research is to obtain a better understanding of the various factors which control one key molecular feature of lycopene - its geometrical configuration - in fresh and processed fruits and vegetables as well as in biological samples. Specifically, the proposed study will seek to:

1. Monitor the physical and chemical state of lycopene in tomato tissues undergoing various thermal treatments.

2. Investigate the impact of production and product formulation variables on the distribution of lycopene geometrical isomers in several commercial and pilot-plant prepared tomato products.

3. Compare the thermal stability of lycopene in red tomatoes to other tomato varieties with unique carotenoid profiles as well as to other fruits and vegetables which are also rich sources of lycopene.

4. Determine the stability of lycopene in different physical states and relative concentrations in an artificial digestion model system.

Ultimately, this study is designed to gain further insights on whether cis isomers of lycopene are formed after biosynthesis and to evaluate the various factors that will influence these isomerization reactions. Understanding the physiochemical characteristics of lycopene as well as various intra and intermolecular processes it undergoes in the native state, during food processing and ultimately in vivo, will provide key insights into the molecule's physical and chemical states at the various stages of its existence from biosynthesis to absorption and distribution.
Chapter I

LITERATURE REVIEW

Carotenoids are a group of over 600 lipid-soluble phytochemicals, commonly known for the brilliant yellow, orange, and red colors they impart in plants and animals (Straub, 1987; Kull and Pfander, 1995). Carotenoids are found in both photosynthetic cells, being complexed with the chlorophylls, as well as in non-photosynthetic tissues. Specific biological functions of carotenoids have been suggested to include protecting plants and algae against photosensitization and acting as accessory pigments in photosynthesis (Krinsky, 1989; Moore et al., 1989; Bendich, 1989; Bendich and Olson, 1989).

Carotenoids are synthesized de novo only in plants. They can be found in animals, however, being responsible for the color of birds, fish, insects, and some invertebrates. Likewise, several animal products such as milk and eggs also contain carotenoids. A number of food color extracts from natural sources such as palm oil, paprika, annatto, and saffron also owe their pigmentation to carotenoids (Borenstein and Bunnel, 1966; Weedon, 1971; Bauernfeind, 1972, 1981). Ultimately, humans and animals rely on their diet as the sole carotenoid source.
The general structure of carotenoids is of the aliphatic or aliphatic-alicyclic type as their chromophoric systems contain several conjugated carbon-carbon double bonds, making them soluble in fats and lipids and yellow to red in color. In addition to the ability to absorb visible light with their chromophore, the large number of double bonds within the carotenoid molecule also renders these compounds very susceptible to isomerization – resulting in shape and color changes as well as in oxidation - degrading into colorless products. Carotenoids have aroused a lot of interest in recent years not only due to their fascinating colors and intriguing chemical structure but also due to emerging evidence of biological and physiological importance beyond their traditional role as vitamin A precursors. This review will address key structural and functional characteristics of carotenoids, their biosynthesis and occurrence in nature along with factors affecting their stability, chemistry and biological functionalities. Special emphasis will be given to the tomato carotenoid lycopene, the impact of fruit and vegetable thermal processing and oxidizing conditions on its physiochemical stability.

1.1. Carotenoids in Fruits and Vegetables

1.1.1. Structure and Nomenclature

Carotenoids can generally be caterogized into two groups: hydrocarbons (carotenes) and their oxygenated derivatives (xanthophylls). A typical carotenoid molecule consists of eight isoprenoid units. These five-carbon units are arranged such that the molecule has a geometric center in the middle (Figure 1.1). Approximately 370 of the 600 naturally occurring carotenoids are chiral, bearing from one to five asymmetric
A. Basic carotenoid skeleton, comprising of 8 isoprenoid units

B. Numbering scheme for carotenoids, lycopene’s structure shown

C. β-carotene

D. Ψ end group  E. β end group  F. ε end group

G. γ end group  H. Κ end group  I. Φ end group

J. χ end group  K. R group

Figure 1.1. General structural characteristics of carotenoids.
carbon atoms. All carotenoids may be chemically derived from the acyclic forty-carbon backbone through cyclization, dehydrogenation, hydrogenation, or oxidation as well as any combination of these processes. Other carotenoids can also arise from rearrangements or degradations of the carbon skeleton itself, as long as the two central methyl groups are retained (Eugster, 1995).

Common and semisystematic names for the majority of the naturally-isolated carotenoids can be found in Key to Carotenoids (Pfander, 1987) and in the Appendix of Carotenoids, Volume 1A (Kull and Pfander, 1995). The latter also provides literature references for spectroscopic properties of carotenoids. In the case of common carotenoids such as β-carotene and lycopene, trivial names are usually employed even without reference to its systematic name.

The International Union of Pure and Applied Chemistry (IUPAC) and IUPAC-International Union of Biochemists (IUB) Commissions on Nomenclature (1975) have established rules for the nomenclature of carotenoids. Key aspects of these rules are as followed:

1. The name of a specific carotenoid is based on the root word “carotene” with two Greek letter prefixes cited in alphabetical order. The prefixes correspond to the chemical nature of the two C₉ terminal groups. Beta (β) and epsilon (ε) refers to cyclohexene type such as β-carotene, while phi (ϕ) and chi (χ) denote aryl groups. Gamma (γ), kappa (κ), and psi (ψ), likewise, represent methylene cyclohexane, cyclopentane, and acyclic groups, respectively (Figure 1.1).
2. The xanthophylls require the additional application of organic chemical nomenclature, incorporating hydroxy, methoxy, carboxy, oxo, and epoxy functional groups names. The R/S convention is applied to designate the absolute configuration at chiral centers.

3. Finally, the possibility of cis-trans isomerism of the carbon-carbon double bonds requires that the cis configuration be denoted for differentiation. In this case, the E/Z convention is used while applying the priority designation rules of the R/S system (Weedon and Moss, 1995).

Figure 1.2 illustrates the application of some of the rules discussed above while depicting the diversity in structural differences among common dietary carotenoids.

1.1.2. Provitamin A Activity of Carotenoids

Vitamin A, an essential nutrient, plays a role in a variety of biological processes including vision, cellular growth and differentiation, reproduction as well as the integrity of the immune system (Olson, 1994). Carotenoids have been reported to contribute up to 50% of vitamin A intake in the American diet (Tee, 1992; Block, 1994). The ability to function as vitamin A precursors is known to be limited to those carotenoids in the diet with a retinoid backbone and an unsubstituted β-ionone group as in the case of β-carotene and α-carotene (Bauernfeind, 1972; Brubacher and Weiser, 1985). Table 1.1 categorizes some of these dietary carotenoids and their predominant isomers into chemical class while denoting whether or not each of these compounds possess provitamin A activity (Deuel et al., 1944a; 1944b; 1945; Tang and Krinsky, 1993; Khachik et al., 1998).
Lycopene, $C_{40}H_{56}$
$\psi, \psi$-carotene

Phytoene, $C_{40}H_{64}$
15-Z-7, 8, 11, 12, 7', 8', 11', 12'-octahydro-$\psi-\psi$-carotene

Phytol, $C_{40}H_{62}$
8'-hexahydro-$\psi, \psi$-carotene

$\zeta$-Carotene, $C_{40}H_{60}$
7, 8, 7', 8'-tetrahydro-$\psi, \psi$-carotene

Neurosporene, $C_{40}H_{58}$
7, 8-dihydro-$\psi, \psi$-carotene

$\gamma$-Carotene, $C_{40}H_{56}$
$\beta, \psi$-carotene

$\alpha$-Carotene, $C_{40}H_{56}$
6'R-$\beta, \epsilon$-carotene

$\beta$-Carotene, $C_{40}H_{56}$
$\beta, \beta$-carotene

$\alpha$-Cryptoxanthin, $C_{40}H_{56}O$
3R, 6'R-$\beta, \epsilon$-caroten-3-ol

$\beta$-Cryptoxanthin, $C_{40}H_{56}O$
3R-$\beta, \beta$-caroten-3-ol

Lutein, $C_{40}H_{56}O_2$
3R, 3'R, 6'R-$\beta, \epsilon$-carotene-3,3'-diol

Zeaxanthin, $C_{40}H_{56}O_2$
3R, 3'R-$\beta, \beta$-carotene-3,3'-diol

Lactucaxanthin, $C_{40}H_{56}O_2$
3S, 6S, 3'S, 6'S-$\epsilon, \epsilon$-carotene-3,3'-diol

Figure 1.2. Semisystematic names, chemical and structural formulas of common dietary carotenoids.
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</tr>
<tr>
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</tr>
<tr>
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</tr>
<tr>
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</tr>
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<td>20. Z Phytofluene</td>
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<td>22. 13, 13'-Z Lutein</td>
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</tr>
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<td>29. 15-Z Zeaxanthin</td>
<td>Dihydroxycarotenoids</td>
<td>No</td>
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</tbody>
</table>

Table 1.1. Dietary carotenoids in humans. Adapted from Khachik et al., 1998.
The biological conversion of carotenoids to vitamin A is believed to be similar in humans and animals, occurring mainly in the intestinal mucosa and the liver. One possible metabolic pathway of provitamin A carotenoids is via the central cleavage mechanism involving the enzyme β-carotene 15, 15’ dioxygenase (EC 1.13.11.21) to yield retinol (Ganguly and Sastry, 1985; Olson, 1994). Another mechanism for conversion has been suggested to proceed via excentric or random cleavage which results in retinal as well as other apocarotenals (Glover, 1960; Sharma et al., 1977; Wang, 1994).

### 1.1.3. Biosynthesis of Lycopene and Other Tomato Carotenoids

Carotenoid syntheses including that of lycopene are extensively discussed in a number of books, symposia proceedings and review articles (Goodwin, 1952, 1980; Hulme, 1970, 1971; Czygan, 1980; Britton, 1983). Carotene biosynthesis in higher plants was reviewed by Jones and Porter (1986) while *in vitro* carotenoid biosynthesis was discussed by Bramley (1985). The overall characteristics of carotenogenesis are similar in higher plants, algae, fungi, and bacteria. This section discusses lycopene synthesis in higher plants using the tomato fruit system as a model.

Carotenogenesis consists primarily of the following stages (Figure 1.3):

1. Formation of mevalonic acid (MVA).
2. Formation of isoprene precursor, isopentenyl pyrophosphate (IPP)
3. Formation of geranylgeranyl pyrophosphate (GGPP)
4. Formation of phytoene
5. Desaturation of phytoene
6. Formation of lycopene
7. Cyclization
8. Formation of xanthophylls
Mevalonic Acid (MVA)

Isopentenyl Pyrophosphate (IPP)

Dimethylallyl Pyrophosphate (DMAPP)

Geranylgeranyl Pyrophosphate

Phytoene

Phytofluene

ζ-Carotene

Neurosporene

Lycopene

γ-Carotene

β-Carotene

Zeaxanthin

Figure 1.3. Biosynthesis of lycopene and selected carotenoids.
Lycopene synthesis involves the first six steps. As is the case with all carotenogenesis, lycopene biosynthesis begins with the reduction of β-hydroxy-β-methyl glutaryl CoA derived from the condensation reaction between three molecules of acetyl-CoA. Isopentenyl pyrophosphate (IPP) serves as the biological isoprene precursor of carotenoids and terpenoids. It is derived from mevalonic acid (MVA). MVA is phosphorylated by kinases and ATP into mevalonic acid 5-pyrophosphate and 5-pyrophosphate successively. MVA 5-pyrophosphate undergoes decarboxylation, yielding the isoprene unit IPP. The enzyme prenyl transferase then catalyzes the chain elongation from C$_5$ isopentenyl pyrophosphate (IPP) to C$_{20}$ geranylgeranyl pyrophosphate (GGPP) (Gross, 1991).

Two molecules of GGPP subsequently give rise to the first C$_{40}$ compound in the biosynthetic pathway, phytoene, via a condensation reaction. Phytoene is eventually converted through a stepwise desaturation process in which the C$_{40}$ chromophore is extended by two double bonds alternatively from both sides of the molecule in each step. This dehydrogenation process brings about phytofluene, ζ-carotene, neurosporene, and finally lycopene, respectively. Ultimately, lycopene serves as the basic substrate for the formation of most cyclic carotenoids via the formation of either β-ring or ε-ring at the terminal ends (Figure 1.4). Following cyclization, the insertion of oxygen functional groups such as hydroxy and epoxy produces xanthophylls such as lutein and zeaxanthin. Hydroxylation is most common at the C-3 position but other positions of the ring or within the chain is susceptible to this and other oxidation reaction as well (Gross, 1991).
Figure 1.4. General scheme for the biosynthesis of common $\beta$ and $\varepsilon$-ring cyclic carotenes. Adapted from Britton, 1983 and Gross, 1991.
1.1.4. Tomato Varieties with Unique Carotenoids Profile

While tomatoes represent the most common source of lycopene, some varieties of tomatoes have a special biosynthetic capacity to produce other carotenoids which are either unique or in altered relative abundance. Mutant varieties such as *Golden jubilee* and *Tangella* have the capacity to biosynthesize several *cis* isomers of lycopene which are not commonly found in other varieties (Zechmeister *et al.*, 1941; Raymundo and Simpson, 1972; Glass and Simpson, 1976). In these varieties, the predominant carotenoid is prolycopene, a tetra-*cis* isomer of lycopene. Figure 1.5 illustrates the biosynthetic pathway of prolycopene from 15-*cis* phytotene.

Compared to all-trans lycopene, prolycopene has two hindered *cis* double bonds (Clough and Pattenden, 1979; Englert *et al.*, 1979), and a shortened chromophore, imparting an orange color in these tomato fruits and hence the common name, Tangerine tomatoes. Zechmeister *et al.* (1943) reported spectroscopic characteristics of prolycopene to have an absorption maximum at 435 nm in hexane and a spectrum void of fine structure in contrast to those observed for lycopene and its mono-*cis* isomers (Figure 1.6). In addition to having an absorption maximum shifted 35 nm upfield in wavelength from all-trans lycopene at 470 nm, prolycopene also has a much lower extinction coefficient of 102,900 AU compared to 184,000 AU for its all-trans counterpart. Prolycopene has also been reported to be present in roots of *Brassica rutabaga* and *Brassica napus* (Joyce, 1954, 1959).
Figure 1.5. Formation of Prolycopene via the conversion of Phytoene in the tangerine mutant of *Lycopersicum esculentum*. Adapted from Britton, 1983, and Goodwin, 1993.
Figure 1.6. Ultraviolet-visible spectrum of lycopene and selected geometrical isomers. Spectrum derived from Waters 996 Photodiode Array Detector @ 2 spectra per second and 1.2 nm resolution.
Several other minor carotenoids have also been isolated and identified in normal and mutant varieties of tomatoes. Zechmeister and Cholnoky (1936) reported the presence of the monohydroxy and dihydroxy derivatives of lycopene — lycoxanthin (\(\psi, \psi\)-caroten-16-ol) and lycophyll (\(\psi, \psi\)-carotene-16, 16'-diol). Both of these lycopene xanthophylls have been identified by other researchers (Went et al., 1942; Curl, 1961, Ben-Aziz et al., 1973) in tomatoes, in Marzano tomatoes (Edwards and Reuter, 1967) and cherry tomatoes (Laval-Martin et al., 1975). The chemical structures for these carotenoids are shown in Figure 1.7 as reported by Markham and Liaaen-Jensen (1968) in addition to a series of epoxides of lycopene and its more saturated precursors, phytoene, phytofluene, and \(\zeta\)-carotene (Britton and Goodwin, 1969; Ben-Aziz et al., 1973). The majority of the epoxides are 1,2-epoxy derivatives. However, the 5, 6-epoxide of lycopene has also been detected along with apoderivatives of lycopene, namely apo-6'lycopeneal and apo-8'lycopenal.
Figure 1.7. Chemical structures of selected minor tomato carotenoids.
1.1.5 Genetics of Tomato Carotenoid Biosynthesis.

The color of tomato fruit is dictated by genes, which adhere to Mendelian inheritance principles. Yellow tomato varieties, for example, are different from their red counterpart by only one recessive gene, \( r \) (Kirk and Tilney-Bassett, 1967; Giuliano et al., 1993). Red tomatoes have alleles of \( r^+ / r^+ \) or \( r^+ / r \) compared to the homozygous recessive alleles \( r/r \) in yellow tomatoes. As a result of this difference, yellow tomatoes lack phytoene and lycopene and are pale in color. Furthermore, changes in a single gene in certain mutant varieties of tomatoes can dramatically alter the carotenoid composition and likewise the color of these fruit. For example, the expression of the dominant gene, \( Del \), yields an increase in the activity of the enzyme lycopene epsilon-cyclase, which converts lycopene to \( \delta \)-carotene. The accumulation of \( \delta \)-carotene at the expense of lycopene makes the former the predominant carotenoid while changing the fruit color from red to orange (Tomes, 1967, 1969; Ronen et al., 1999). Similarly, the allele \( B \) produces high-\( \beta \)-carotene tomato fruit that also appear orange in color. The Tangerine-type tomato, as discussed earlier, is rich in prolycopene, proneurosporene, and \( \zeta \)-carotene, being homozygous fruits with \( t/t \) (Khudairi, 1972). Figure 1.8. depicts a generalized scheme of the genetics of tomato carotenoid biosynthesis.
Mevalonic Acid

\[ hp+/hp \rightarrow (Overall \ Level \ of \ Carotenoids) \]

Geranylgeranyl Pyrophosphate

\[ r+/r \rightarrow (Red \ vs. \ Yellow) \]
\[ at+/at \rightarrow (Red \ vs. \ Apricot) \]

Phytoene

\[ gh+/gh \rightarrow (Red \ vs. \ Ghost) \]

\[ \delta-\text{Carotene} \]
\[ \alpha-\text{Zeaxcarotene} \]
\[ \text{Del}+/\text{Del} \]
\[ B+/B \]
\[ \beta-\text{ZeaxCarotene} \]
\[ \gamma-\text{Carotene} \]
\[ \beta-\text{Carotene} \]

\[ \zeta-\text{carotene} \]
\[ \text{Neurosporene} \]
\[ \text{Proneurosporene} \]
\[ \text{Lycopene} \]
\[ \text{Prolycopene} \]

\[ t+/t \rightarrow (Red \ vs. \ Tangerine) \]

Figure 1.8. Genetics of carotenoid biosynthesis in tomatoes. Adapted from Khudairi, 1972.
1.2. Chemical and Biological Properties of Lycopene:

The relevance of carotenoids to human nutrition and health was historically confined to those possessing pro-vitamin A activity such as α-carotene and β-carotene. However, other carotenoids have also emerged as important dietary phytochemicals. Among these carotenoids with potentially beneficial biological activities beyond their traditional role as vitamin A precursors, lycopene in particular is one with promising implications for human nutrition and health.

This section of the review will examine lycopene’s natural occurrence, stability, bioavailability, and physiological distribution to gain perspective on the increasing interest in this compound. Special emphasis will be given to key aspects of isolation and analytical protocols, the impact of food thermal processing on lycopene physiochemical stability, and research findings on its potential role in human health.

1.2.1. Natural Occurrence of Lycopene

Of the more than 50 dietary carotenoids, lycopene, found primarily in tomatoes and tomato products, is the most prevalent in the Western diet and the most abundant in human serum. It was first isolated by Hartsen (1873) from *Tamus communis* L. berries as a deep red crystalline pigment. Millardet (1875) obtained a crude mixture containing lycopene from tomatoes, referring to it as *solanorubin*. Duggar (1913) referred to lycopene as *lycopersicon* in his work detailing the effects of growth conditions on its development. Schunck (1903) gave lycopene its name after showing that this pigment from tomato had a different absorption spectrum than carotenes from carrots.
In the common variety of tomatoes, *Lycopersicon esculentum*, lycopene is found predominantly in the all-\textit{trans} configuration (Zechmeister \textit{et al.}, 1941) and in concentrations of 3.1—7.7 mg/100 g of ripe fruit. In certain varieties, such as *Lycopersicon pimpinellifolium*, levels as high as 40 mg/100 g of tissue have been reported, accounting for 95—100% of the total carotenoid content of these tomatoes (Porter and Lincoln, 1950). Recent advances in isolation and chromatographic separation methodologies have shown that lycopene is much more widely distributed in nature than once thought (Nguyen and Schwartz, 1999). Table 1.2 lists botanical and food sources of lycopene.

Lycopene is found predominantly in the chromoplast of plant tissues. In tomatoes, lycopene biosynthesis increases dramatically during the ripening process as chloroplasts undergo transformation to chromoplasts (Kirk and Tilney-Bassett, 1978). Laval-Martin (1974) categorized tomato chromoplasts into two types. Globulous chromoplasts containing mainly β-carotene are found in the jelly part of the pericarp, while chromoplasts in the outer part of the pericarp contain voluminous sheets of lycopene. The development and ultrastructure of these sheets of lycopene were studied by Ben-Shaul and Naftali (1969) and named crystalloids. Mohr (1979) noted that in both normal red and high-lycopene varieties, the development of the pigment bodies are similar, following the same sequence of granal membrane loss, globule size and density increase, and deposition of crystal bodies along the extended thylakoid system.
<table>
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<tr>
<th>Common Name</th>
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Table 1.2. Plant sources of lycopene (cont.)

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<td>28.</td>
<td>Persimmon</td>
<td>Fruit</td>
<td><em>Diospyros kaki</em></td>
</tr>
<tr>
<td>29.</td>
<td>Plum</td>
<td>Fruit</td>
<td><em>Prunus domestica</em></td>
</tr>
<tr>
<td>30.</td>
<td>Pumpkin</td>
<td>Fruit</td>
<td><em>Cucurbita pepo</em></td>
</tr>
<tr>
<td>31.</td>
<td>Ramanas rose</td>
<td>Plant</td>
<td><em>Rosa rugosa</em></td>
</tr>
<tr>
<td>32.</td>
<td>Red bryony</td>
<td>Plant</td>
<td><em>Bryonia dioica</em></td>
</tr>
<tr>
<td>33.</td>
<td>Rosa mosqueta</td>
<td>Plant</td>
<td><em>Rosa rubiginosa</em></td>
</tr>
<tr>
<td>34.</td>
<td>Rose</td>
<td>Fruit</td>
<td><em>Rosa canica</em></td>
</tr>
<tr>
<td>35.</td>
<td>Rutabaga</td>
<td>Root</td>
<td><em>Brassica napus var. napobrassica</em></td>
</tr>
<tr>
<td>36.</td>
<td>Saffron</td>
<td>Seed</td>
<td><em>Crocus sativus</em></td>
</tr>
<tr>
<td>37.</td>
<td>Sallow thorn</td>
<td>Fruit</td>
<td><em>Hippophae rhamnoides</em></td>
</tr>
<tr>
<td>38.</td>
<td>Tea</td>
<td>Leaf</td>
<td><em>Camellia sinensis</em></td>
</tr>
<tr>
<td>39.</td>
<td>Tomato</td>
<td>Fruit</td>
<td><em>Lycopersicon esculentum</em></td>
</tr>
<tr>
<td>40.</td>
<td>Turnip</td>
<td>Root</td>
<td><em>Brassica rapa var. rapa</em></td>
</tr>
<tr>
<td>41.</td>
<td>Watermelon</td>
<td>Fruit</td>
<td><em>Citrullus lanatus</em></td>
</tr>
<tr>
<td>42.</td>
<td>Yew</td>
<td>Fruit</td>
<td><em>Taxus baccata</em></td>
</tr>
</tbody>
</table>

From:
- Joyce (1954, 1959),
- List and Horhammer (1979),
- Harborne and Baxter (1983),
- Godoy and Rodriguez-Amaya (1987),
- Variyar and Bandyopadhyay (1990),
- Gross (1991),
- Duke (1992),
- Mangels *et al.* (1993),
- Wilberg and Rodriguez-Amaya (1995),
- Choo *et al.* (1996),
- Cano *et al.* (1996),
- Muller (1997),
- Duke and Beckstrom-Sternberg (1998),
1.2.2. Isolation and Chromatographic Separation

The basic chemical information on lycopene is fairly complete, with a research history dating back to the beginning of this century. The molecular formula of lycopene, $\text{C}_{40}\text{H}_{56}$, was first determined when Willstatter and Escher (1910) presented their work showing that lycopene is an isomer of the carotenes. Karrer et al. (1930) published the chemical structure of lycopene, which was subsequently confirmed by Kuhn and Grundmann (1932) by identifying its degradation products following chromic acid oxidation.

Zechmeister and coworkers made significant progress toward isolation of lycopene, determination of spectrophotometric properties via iodine-catalyzed stereomutation and establishment of the foundation for a better understanding of lycopene's chemical stability in terms of isomerization and oxidation (Zechmeister, 1962; Zechmeister and Cholnoky, 1936; Zechmeister and Polgar, 1944; Zechmeister and Tuzson, 1938a, b; Zechmeister et al., 1941, 1943). Many of these basic techniques and the fundamental considerations for the isolation, handling, and separation of lycopene they addressed are still applicable today.

The general structure of lycopene is an aliphatic hydrocarbon with 11 conjugated carbon–carbon double bonds, making it soluble in fats and lipids and red in color. Being acyclic, lycopene possesses symmetrical planarity and has no vitamin A activity. As a highly conjugated polyene, it is particularly susceptible to oxidative degradation. Physical and chemical factors known to degrade other carotenoids, including elevated temperature, exposure to light, oxygen, extremes in pH, and active surfaces (Davies,
1976; Moss and Weedon, 1976; Scita, 1992; Crouzet and Kanasawud, 1992), apply to lycopene as well. Additionally, increases in temperature increase the rate of degradative reactions (Henry et al., 1998).

Another type of change that lycopene undergoes readily as a polyene is cis-trans isomerization. With very few exceptions, lycopene from natural plant sources exists predominantly in the all-trans configuration, the most thermodynamically stable form (Zechmeister et al., 1941; Wilberg and Rodriguez-Amaya, 1995; Emenhiser et al., 1995). As a result of the 11 conjugated carbon–carbon double bonds in its backbone, lycopene can theoretically assume $2^{11}$ or 2,048 geometrical configurations. While a large number of geometrical isomers are theoretically possible for all-trans lycopene, according to Pauling (1939) and Zechmeister et al. (1941), only certain ethylenic groups of a lycopene molecule can participate in cis-trans isomerization because of steric hindrance. In fact, only about 72 lycopene cis isomers are structurally favorable (Zechmeister, 1962).

The interconversion of lycopene geometrical configuration can be induced in vitro using reagents such as iodine which will release the $\pi$-bond of carbon atoms involved in double bonds. The free rotation about the axis of the remaining single $\sigma$-bond between these carbons, carrying substituted groups, and subsequent reformation of the double bond results in a change in configurational geometry (Zechmeister and Tuxson, 1939). Fig. 1.9 illustrates the structural distinctions of the predominant lycopene geometrical isomers. As has been discussed in earlier section, by applying rules of designation similar to those involving $R$ and $S$ chirality, the stereochemical prefixes $E$
all-trans lycopene

15-cis lycopene

13-cis lycopene

11-cis lycopene

9-cis lycopene

7-cis lycopene

5-cis lycopene

7,9,9',7'-cis lycopene

Figure 1.9. Lycopene geometrical isomers. Structure A is the predominant species in tomatoes and tomato products (~95%). Structures B, C, E, and G are found in human serum, comprising more than half of the total lycopene content. Structure H (Zechmeister, 1941) is the naturally occurring form of lycopene in Tangerine-type tomatoes.
and Z are used whenever the corresponding trans and cis designations are not sufficient to denote the priority of the two substituents on the individual carbon atoms involved in the double bond (Weedon and Moss, 1995). All-trans lycopene and its 5-cis geometrical isomer, therefore, can also be referred to as the all-E and 5-Z configurations. Likewise, the all-trans configuration is implied in the absence of a geometrical designation (IUPAC, 1975).

Cis-isomers of lycopene have physical and chemical properties distinctly different from their all-trans counterpart. Some of the differences observed as a result of a trans to cis isomerization reaction include decreased color intensity, lower melting points, smaller extinction coefficients, a shift in the $\lambda_{\text{max}}$, and the appearance of a new maximum in the ultra-violet spectrum (Zechmeister and Polgar, 1944). The decrease in color intensity is of paramount importance as far as quality perception is concerned. Smaller extinction coefficients for cis isomers of lycopene need to be taken into account during quantitative analysis of lycopene isomers to avoid under-estimation. The appearance of the new maxima in the ultraviolet region, so-called “cis-peaks,” and their relative intensity are useful in assigning tentative identification of lycopene isomers.

Lycopene’s extraction, storage, handling, and analysis have to be carried out under controlled environmental factors not only to minimize oxidative degradation but also to avoid the introduction of artifactual levels of isomers. Exposure of extracted lycopene to light should be avoided, and only gold, yellow (Landers and Olson, 1986), or red lights should be used. Antioxidants such as butylated hydroxytoluene (BHT)
should be employed in extraction and separation solvents to control oxidation and isomerization reactions of lycopene (Nguyen and Schwartz, 1998a). In addition, nitrogen or argon headspace can be employed to keep exposure to atmospheric oxygen to a minimum. Saponification, using methanolic potassium hydroxide, can be performed to enhance lycopene's analysis by eliminating chlorophyll and lipid materials, which can interfere with its chromatographic elution and detection (Kimura et al., 1990).

High-performance liquid chromatography (HPLC) is the conventional separation method of choice for lycopene. In general, lycopene is separated from other carotenoids using reversed-phase C18 columns. Variations in the properties of the silica packing material in terms of particle size, porosity, carbon load, end-capping technique, and polymerization can greatly influence the sensitivity and selectivity of lycopene analysis (Sander and Wise, 1987; Craft, 1992; Epler et al., 1992; Sander et al., 1994).

To separate lycopene isomers, however, reversed-phase C30 stationary phase is often employed to achieve superior selectivity of lycopene isomers compared to conventional C18 reversed-phase and silica normal-phase columns (Sander et al., 1994; Emenhiser et al., 1996). The polymerically synthesized C30 columns not only provide excellent separation of the all-trans lycopene isomers from the cis counterpart, but they also exhibit remarkable selectivity among the individual cis isomers themselves (Emenhiser et al., 1996; Rouseff et al., 1996). Recently, another HPLC method using multiple columns in series has also been shown to comparably resolve cis and trans lycopene isomers (Schierle et al., 1997).
1.2.3. Stability During Food Processing

Since lycopene is responsible for the red color of tomatoes and color is used as an index of quality for tomato products, minimizing the loss of lycopene throughout the production process and during storage has always been important. As discussed earlier, the loss of lycopene can be attributed to both oxidation and isomerization. Cole and Kapur (1957a, b) examined the kinetics of lycopene degradation by studying the effects of oxygen, temperature, and light intensity on the formation of its volatile oxidation products. Adding to Monselise and Berk's (1954) report of oxidative degradation of lycopene in heat-treated tomato puree, Cole and Kapur (1957b) reported significant losses of lycopene in serum-free tomato pulp samples following thermal treatment at 100°C in the presence of oxygen, with or without light. The intensity of illumination and temperature were found to be in direct correlation with lycopene degradation in the presence of oxygen.

Noble (1975) found that heat concentration of tomato pulp resulted in approximately 57% loss of lycopene. Sharma and Le Maguer (1996) reported the kinetics of lycopene degradation in tomato pulp solids to be a pseudo first-order reaction. Boskovic (1979) observed a reduction of all-trans lycopene content by up to 20% following processing and extended storage of dehydrated tomato products.

In other lycopene-containing fruits, such as papaya slices, food processing operations, such as freezing and canning, led to a significant decrease in total carotenoid content, of which lycopene is a major component (Cano et al., 1996). In contrast, a number of studies on the thermal stability of carotenoids in processed fruits and vegetables have found that hydrocarbon carotenoids such as lycopene, \( \alpha \)-carotene, and
β-carotene are relatively heat-resistant (Khachik et al., 1992a, b). According to Khachik et al. (1992a), most of these carotenoids remain stable following bench-top food preparation. It is difficult to reconcile the differences in these findings, as a number of factors may contribute to the difficulties in accounting for the changes in lycopene levels. The inability to distinguish between chemical degradation and geometrical isomerization of the parent molecule, for example, is a common limitation.

Isomerization of fruit and vegetable carotenoids as a result of thermal treatments during food processing and preparation are well-known, especially for β-carotene (Panalaks and Murray, 1970; Sweeney and Marsh, 1971; Bushway, 1985; Tsukida et al., 1981; Quackenbush, 1987; Chandler and Schwartz, 1988; Lessin et al., 1997). For example, Lessin et al. (1997) reported that canning fresh tomatoes increases the β-carotene cis isomer content from 12.9% to 31.2%. Heating tomato juice (Stahl and Sies, 1992) and bench-top preparation of a spaghetti sauce from canned tomatoes (Schierle et al., 1997) were shown to increase the level of lycopene cis isomers. However, Khachik et al. (1992b) indicated that common heat treatments during food preparation such as microwaving, boiling, steaming, and stewing did not significantly alter the carotenoid distribution in green vegetables and tomatoes. Other studies have also reported the levels of lycopene cis isomers in thermally processed tomato products to be low (Clinton et al., 1996; Emenhiser et al., 1996).

Lycopene isomer profiles in dietary and biological samples (Fig. 1.10) show similar all-trans to cis isomer ratios in fresh tomato and processed tomato soup samples, while human serum and prostate tissue distributions have a much higher cis isomer content. Experimental data from our laboratory have recently confirmed that in tomato
Fig. 1.10. Representative chromatograms illustrating *cis* and *trans* isomers of lycopene in fresh tomato, tomato soup, human serum, and human prostate. Reprinted from Clinton *et al.* (1996).
products of varying moisture content, fat content, and container type, thermal treatments
during usual food preparation or commercial production processes do not result in
significant losses of lycopene or a shift in the distribution of cis-lycopene isomers
(Nguyen and Schwartz, 1998a). Thus, lycopene in the diet from both fresh and
processed foods is consumed predominantly as the all-trans configuration. The elevated
levels of cis isomers observed in human biological samples cannot be attributed to
consumption of thermally processed food but rather to in-vivo mechanisms, which are
still unclear at this time.

A great deal of information remains to be gathered on the thermal behavior of
lycopene before definitive answers can be provided regarding its physical state and
stability. Nonetheless, lycopene is more stable in native tomato fruit tissues and
matrices than in isolated or purified form (Simpson et al., 1976), as a result of the
protective effects of cellular constituents such as water. Therefore, care must be
exercised to minimize the loss of lycopene through oxidation or isomerization during
extraction, storage, handling, and analysis to accurately account for cause-and-effect
changes. Table 1.3. lists some common food sources of lycopene, taking serving sizes
into consideration.
<table>
<thead>
<tr>
<th>Food</th>
<th>Type</th>
<th>Amount per serving (mg)</th>
<th>Amount per 100 g wet wt.</th>
<th>Serving size</th>
</tr>
</thead>
<tbody>
<tr>
<td>Apricots</td>
<td>Fresh</td>
<td>0.005&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.007</td>
<td>140 g</td>
</tr>
<tr>
<td>Apricots</td>
<td>Canned, drained</td>
<td>0.065&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.091</td>
<td>140 g</td>
</tr>
<tr>
<td>Apricots</td>
<td>Dried</td>
<td>0.86&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.34</td>
<td>40 g</td>
</tr>
<tr>
<td>Chili</td>
<td>Processed</td>
<td>1.08-2.62&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.40-3.41</td>
<td>130 g</td>
</tr>
<tr>
<td>Grapefruit</td>
<td>Pink, fresh</td>
<td>3.36&lt;sup&gt;a&lt;/sup&gt;</td>
<td>4.70</td>
<td>140 g</td>
</tr>
<tr>
<td>Guava</td>
<td>Pink, fresh</td>
<td>5.40&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.56</td>
<td>140 g</td>
</tr>
<tr>
<td>Guava juice</td>
<td>Pink, processed</td>
<td>3.34&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8.35</td>
<td>240 ml ~ 250 g</td>
</tr>
<tr>
<td>Ketchup</td>
<td>Processed</td>
<td>16.60&lt;sup&gt;a&lt;/sup&gt;</td>
<td>3.32</td>
<td>1 tbsp. ~ 20 g</td>
</tr>
<tr>
<td>Papaya</td>
<td>Red, fresh</td>
<td>2.00-5.30&lt;sup&gt;b&lt;/sup&gt;</td>
<td>2.8-7.42</td>
<td>140 g</td>
</tr>
<tr>
<td>Pizza sauce</td>
<td>Canned</td>
<td>12.71&lt;sup&gt;a&lt;/sup&gt;</td>
<td>15.89</td>
<td>125 g</td>
</tr>
<tr>
<td>Pizza sauce</td>
<td>From pizza</td>
<td>32.89&lt;sup&gt;c&lt;/sup&gt;</td>
<td>9.867</td>
<td>slice ~ 30 g</td>
</tr>
<tr>
<td>Rosehip puree</td>
<td>Canned</td>
<td>0.78&lt;sup&gt;a&lt;/sup&gt;</td>
<td>0.47</td>
<td>60 g</td>
</tr>
<tr>
<td>Salsa</td>
<td>Processed</td>
<td>9.28&lt;sup&gt;d&lt;/sup&gt;</td>
<td>3.71</td>
<td>2 tbsp. ~ 40 g</td>
</tr>
<tr>
<td>Spaghetti sauce</td>
<td>Processed</td>
<td>17.50&lt;sup&gt;d&lt;/sup&gt;</td>
<td>21.88</td>
<td>125 g</td>
</tr>
<tr>
<td>Tomatoes</td>
<td>Red, fresh</td>
<td>3.1-7.74&lt;sup&gt;c&lt;/sup&gt;</td>
<td>4.03-10.06</td>
<td>130 g</td>
</tr>
<tr>
<td>Tomatoes</td>
<td>Whole, peeled, processed</td>
<td>11.21&lt;sup&gt;c&lt;/sup&gt;</td>
<td>14.01</td>
<td>125 g</td>
</tr>
<tr>
<td>Tomato juice</td>
<td>Processed</td>
<td>7.83&lt;sup&gt;c&lt;/sup&gt;</td>
<td>19.58</td>
<td>240 mL ~ 250 g</td>
</tr>
<tr>
<td>Tomato soup</td>
<td>Canned, condensed</td>
<td>3.99&lt;sup&gt;e&lt;/sup&gt;</td>
<td>9.77</td>
<td>245 g</td>
</tr>
<tr>
<td>Tomato paste</td>
<td>Canned</td>
<td>30.07&lt;sup&gt;c&lt;/sup&gt;</td>
<td>9.02</td>
<td>30 g</td>
</tr>
<tr>
<td>Watermelon</td>
<td>Red, fresh</td>
<td>4.10&lt;sup&gt;a&lt;/sup&gt;</td>
<td>11.48</td>
<td>280 g</td>
</tr>
<tr>
<td>Vegetable juice</td>
<td>Processed</td>
<td>7.28&lt;sup&gt;c&lt;/sup&gt;</td>
<td>17.47</td>
<td>240 mL ~ 250 g</td>
</tr>
</tbody>
</table>

Table 1.3. Common food sources of lycopene

<sup>a</sup> From USDA (1998)  
<sup>b</sup> From Mangels et al. (1993)  
<sup>c</sup> From Nguyen and Schwartz (1998a)  
<sup>d</sup> From Nguyen and Schwartz (1999)
1.2.4. Lycopene Absorption and Distribution in Humans

Lycopene follows the same intestinal absorption path as dietary fat since it is very lipophilic. Likewise, as lycopene and β-carotene are both hydrocarbon carotenoids, it is expected that they share similar absorption patterns. Once absorbed, however, β-carotene can follow a different metabolic path as it possesses provitamin A activity. Comprehensive reviews by Wang (1994) and Parker (1996) on β-carotene metabolism are recommended for more details.

Lycopene absorption consists of four main phases: digestion of food matrix, formation of mixed micelles, uptake by intestinal mucosal cells, and transport to the blood stream. Masticative and digestive actions facilitate the release of lycopene from the food matrix while the presence of fat and conjugated bile acids accentuates its absorption. The efficiency of this process, however, is influenced by several factors including the physical state of lycopene in the food matrix, particle size before and after mastication as well as digestive processes (Johnson, 1998).

In the small intestine, ingested lycopene molecules are incorporated into micelles formed via the interaction of dietary lipids, the products of lipid digestion (mono-, di-, and triacylglycerols, fatty acids, cholesterol, and phospholipids) and bile acids (Sies and Stahl, 1998). Micelle formation is necessary for lycopene absorption as absorption of carotenoids is often very low when ingested in the absence of adequate lipid (Olson, 1994; Prince and Frisoli, 1993). Dietary fiber, which has been shown to interfere with micelle formation (Rock and Swenseid, 1992), can also decrease the overall lycopene uptake. The absence of bile or other malfunctioning of the lipid absorption machinery
will also reduce the amount of lycopene being absorbed by the intestinal mucosal cells. Overall, as a fat-soluble compound, lycopene absorption is affected by the presence and amount of dietary lipid as well as by factors influencing lipid absorption itself.

Absorption of lycopene has been suggested to occur via passive transport as mixed micelles come into contact with the intestinal mucosal cells of the duodenum (Erdman et al., 1993). Other mechanisms of absorption have not been suggested, although there is evidence of interactions between lycopene and other carotenoids administered in combined oral doses (Johnson et al., 1997). Figure 1.11 illustrates that following absorption, lycopene is packaged into chylomicrons and transported to the bloodstream via the lymphatics (Johnson, 1998). Similar to most other carotenoids, lycopene molecules, which are not packaged into chylomicrons for distribution, will be lost after about three days upon the sloughing off of mucosal cells into the lumen. In the plasma, lycopene is carried exclusively by lipoproteins and its appearance in lipoprotein fractions follows the same time-course as triacylglycerols ingested in the same meal. Specifically, the majority of the absorbed lycopene molecules are associated with low-density lipoproteins (LDL). Only a minor fraction is found in high-density and very-low-density lipoproteins (Krinsky et al., 1998). Likewise, lycopene distribution among lipoproteins is similar to β-carotene, and has the same relative profile in both sexes (Forman et al., 1998).
Figure 1.11. Schematic of lycopene absorption in the human intestinal epithelial cell.
1.2.5 Carotenoid Bioavailability and the *In Vitro* Digestion Model

Ironically, in the case of lycopene's absorption, food processing is in fact a value-added step, in that more lycopene becomes bioavailable following thermal treatment. Heating of tomato juice was shown to result in an improvement in uptake of lycopene in humans (Stahl and Sies, 1992). Gartner *et al.* (1997) showed that tomato paste, a processed product, has more bioavailable lycopene than fresh tomatoes when both are consumed in conjunction with corn oil. This appears to be the result of thermal disruption and weakening of lycopene-protein complexes as well as the rupturing of cell walls. Likewise, various food processing operations such as chopping and pureeing, which result in a reduction in physical size of food particle, will also enhance the bioavailability of carotenoids including lycopene (Erdman *et al*., 1988; 1993).

Carotenoid's bioavailability, including that of lycopene, is generally assessed by monitoring the postprandial level in the plasma following the administration of either purified carotenoids, natural carotenoid-rich sources or a high-carotenoid meal (Micozzi *et al*., 1992; de *Pee et al*., 1995; Oshima *et al*., 1997). Likewise, other investigators such as Gartner *et al*., (1997) monitored the response in the chylomicron fraction of lycopene after consumption of tomato products. Research studies on the bioavailability of carotenoids are often limited by several factors. The use of human subjects is costly as well as time and labor intensive. In addition, most laboratory animals, with the exception of primates, ferrets and preruminant calves, convert provitamin A carotenoids quite efficiently into retinol, making it difficult to account for carotenoids in terms of mass balance.
The *in vitro* digestion technique represents a rapid and reliable alternative to the use of human and animal subjects to assess the stability as well as relative bioavailability of food components. This technique has been successfully employed for the *in vitro* assessment of the bioavailability of amino acids (Linberg *et al*., 1998), cholesterol (Fouad *et al*., 1991), iron (Gangloff *et al*., 1996), phosphorus (Liu *et al*., 1998), vitamin B6 (Ekanayake *et al*., 1986) as well as of carotenoids (Garret *et al*., 1997). The digestive phases of the procedure simulate in sequential order such gastrointestinal conditions as physiological pH changes and stomach emptying time. Other physiological relevant components of the protocol includes the introduction of digestive enzymes, bile extracts and mechanical agitation, which are patterned after human gastric and duodenal occurrences. At the completion of the digestive phases, this *in vitro* model successfully transferred lipophiles such as carotenoids from the food matrix to the micellar phase. Mixed micelles are considered the only effective way to solubilize and transport lipophilic compounds to the enterocytes *in vivo* (Thompson and Dietschy, 1981).

The use of the Caco-2 epithelial cell line, additionally, provides a biological reference for the absorption of carotenoids by intestinal mucosal cells. Monolayers of Caco-2 cells have been effectively used to study passive transport of drugs (Audus *et al*., 1990; Lennemas *et al*., 1996), vitamin B12 (Dix *et al*., 1990) and nickel (Tallkvist and Tjalve, 1998) *in vitro*. Caco-2 cells are derived from human adenocarcinoma of the colon (During *et al*., 1998) originally developed from American Type Culture Collection (ATCC) in Rockville, MD. This cell line has been shown to spontaneously
differentiate at confluency into polarized cells with enterocyte-like characteristics (Pinto et al., 1981, Hidalgo et al., 1989). A recently identified subclone of the Caco-2 cell line, TC7, possesses β-carotene 15, 15'-dioxygenase activity (During et al., 1998), presenting an unprecedented opportunity to study the in vitro efficiency of provitamin A carotenoids metabolism and to assess good sources of vitamin A in foods.

While the selections of pH values and incubation times for the in vitro digestion model are optimized, taking into account the effects of the digested meal’s composition and size, they cannot duplicate the in vivo processes exactly. Nonetheless, apical uptake of micellarized carotenoids by Caco-2 cells, currently represents the most biologically relevant in vitro system to study transepithelial transport of carotenoids. The relative bioavailability values from this system can be applied to substantiate findings from human and animal bioavailability studies or to fine-tune the design of future human trials. In this study, the coupling of the digestive protocol with the Caco-2 cell uptake analysis allows for the monitoring of isomer distribution of carotenoids hypothetically destined to be transported to the bloodstream.

1. 2. 6. Distribution of Lycopene in Biological Fluids and Tissues

The carotenoid profile of human serum is quite extensive and complex. At least 18 different carotenoids have thus far been identified in human serum, with β-carotene and lycopene being the prominent carotenoids (Krinsky et al., 1990; Khachik et al., 1992a, 1995). Similarly, Peng and Peng (1992) found lycopene to be the predominant carotenoid present in buccal mucosal cells at 15.54 ng/10^6 cells. The
The advent of new and refined separation and detection technologies has greatly increased the available information regarding the distribution of lycopene in human serum and selected tissues (Bieri et al., 1985; Kaplan et al., 1990; Schmitz et al., 1991; Stahl et al., 1993; Yeum et al., 1996; Clinton et al., 1996). Yeum et al. (1996) reported the distribution of 13 carotenoids and carotenoid isomers in the plasma of both men and women (Table 1.4).

Lycopene has been shown to exist in several geometrical configurations in human plasma and in a variety of tissue samples, where the cis isomer content ranges from 50 to 88% of the total lycopene level (Krinsky et al., 1990; Schmitz et al., 1991; Stahl et al., 1992, 1993; Emenhiser et al., 1996; Clinton et al., 1996). The two most predominant lycopene isomers are the all-trans and its 5-cis counterpart (Schierle et al., 1997).

Lycopene levels in various human organs and tissues have also been studied (Parker, 1988; Kaplan et al., 1990; Schmitz et al., 1991; Nierenberg and Nann, 1992; Stahl et al., 1992). The findings were summarized by Stahl and Sies (1996) and are adapted in Table 1.5. It is important to note the large differences between one type of tissue and another, as well as the relative amounts reported by independent investigators. The latter differences suggest that the absolute value being reported is only meaningful in the context of individual studies and methodologies.

The deposition of lycopene in a variety of tissues reflects the effective transfer from plasma lipoproteins. The mechanism for the deposition and immobilization of carotenoids in adipose and other tissues as well as intracellular transport of carotenoids, however, are poorly understood. Lycopene is found at relatively high concentration in
<table>
<thead>
<tr>
<th>Carotenoid</th>
<th>Young men</th>
<th>Young women</th>
<th>Older men</th>
<th>Older women</th>
</tr>
</thead>
<tbody>
<tr>
<td>α-carotene</td>
<td>0.09 ±0.01</td>
<td>0.18 ±0.04</td>
<td>0.27 ±0.07</td>
<td>0.20 ±0.04</td>
</tr>
<tr>
<td>All-trans-β-carotene</td>
<td>0.44 ±0.07</td>
<td>0.80 ±0.17</td>
<td>1.51 ±0.41</td>
<td>0.78 ±0.10</td>
</tr>
<tr>
<td>13-cis-β-carotene</td>
<td>0.03 ±0.01</td>
<td>0.05 ±0.01</td>
<td>0.09 ±0.02</td>
<td>0.04 ±0.01</td>
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<tr>
<td>All-trans-lycopene</td>
<td>0.38 ±0.05</td>
<td>0.37 ±0.06</td>
<td>0.41 ±0.06</td>
<td>0.28 ±0.02</td>
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<tr>
<td>9-cis-lycopene</td>
<td>0.07 ±0.01</td>
<td>0.07 ±0.01</td>
<td>0.08 ±0.01</td>
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<td>13-cis-lycopene</td>
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<td>0.10 ±0.02</td>
<td>0.14 ±0.02</td>
<td>0.09 ±0.01</td>
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<tr>
<td>15-cis-lycopene</td>
<td>0.01 ±0.001</td>
<td>0.01 ±0.001</td>
<td>0.01 ±0.003</td>
<td>0.01 ±0.00</td>
</tr>
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Table 1.4. Serum concentrations (µmol/L) of selected carotenoid species as quantified by Yeum et al. (1996).
<table>
<thead>
<tr>
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<td>Adipose</td>
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<tr>
<td>Adrenal</td>
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<td>21.60</td>
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<td>—</td>
<td>1.90</td>
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<tr>
<td>Brain</td>
<td></td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>2.55*</td>
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<td>Breast</td>
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<td>—</td>
<td>0.78</td>
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<td>0.43</td>
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<td>Cervix</td>
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<td>Kidney</td>
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<tr>
<td>Liver</td>
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<td>5.72</td>
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<td>—</td>
<td>0.57</td>
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<td>Ovary</td>
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<td>Prostate</td>
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<td>—</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>0.63**</td>
</tr>
<tr>
<td>Skin</td>
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<td>—</td>
<td>—</td>
<td>0.42</td>
<td>—</td>
<td>—</td>
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<td>Stomach</td>
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<td>—</td>
<td>—</td>
<td>0.20***</td>
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<td>—</td>
<td>4.34</td>
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</table>

Table 1.5. Tissue concentrations of lycopene.

* From Craft et al., 1998.
** From Clinton et al., 1996.
*** From Clinton, 1998.
testes and adrenals, where a high rate of LDL uptake has been noted (Spady et al., 1985). Furthermore, Clinton et al. (1996) compared major carotenoids levels in normal vs malignant human prostate tissue. Lycopene and other major carotenoids were reportedly present in higher concentration in the malignant prostate tissue. In general, while lycopene can be found in a large number of human tissues and organs, the relative amounts have not been systematically correlated to the types or functions of these biological tissues.

1.2.7. Biological and Clinical Implications

The majority of studies involving lycopene prior to the past two decades primarily focused on its physical and chemical properties in the context of color stability in food processing. The shift in emphasis to the biological effects of lycopene is a fairly recent phenomenon, with the most dramatic rise in interest occurring after the publication of a number of recent key findings. Di Mascio et al. (1989) discovered that lycopene is the most efficient singlet oxygen quencher of the biological carotenoids, including β-carotene. Levy et al. (1995) showed lycopene to be a more potent inhibitor of human cancer cell proliferation than either α-carotene or β-carotene. An epidemiological collaboration to these laboratory findings was provided by the Harvard Health Professionals Follow-Up Study (Giovannucci et al., 1995), in which the relationship between intake of various carotenoids, retinol, fruits, and vegetables and the reduced risk of prostate cancer was examined for a cohort of 47,894 male subjects. Giovannucci et al. (1995) concluded that consumption of fresh tomatoes, tomato sauce,
and pizza, which account for the bulk of dietary lycopene intake, is significantly related to a lower incidence of prostate cancer.

Prior to the latter study, accumulated human epidemiological evidence indicated that diets high in tomatoes may reduce the risk of developing cervical, colon, esophageal, rectal, and stomach cancers (Bjelke, 1974; Cook-Mozaffari et al., 1979; Tajima and Tominaga, 1985; Batieha et al., 1993; Ramon et al., 1993; Potischman et al., 1994). In another case-control study, a high intake of fresh tomatoes was linked to a protective effect of the digestive tract against the risk of cancer (Franceschi et al., 1994). A high tomato intake in an elderly American population was likewise associated with a 50% reduction in mortality from cancers at all sites (Colditz et al., 1985).

Palan et al. (1996) reported a decrease in plasma lycopene level, along with β-carotene, canthaxanthin, retinol, and α-tocopherol, in women from New York City diagnosed with cervical intraepithelia neoplasia (CIN) or cervical cancer. The authors suggested that the lowered plasma antioxidant levels might play a role in the pathogenesis of CIN and carcinoma of the cervix. Similarly, Ha et al. (1996) observed lowered plasma lycopene in patients with chronic renal failure compared to the control group.

Rao and Agarwal (1998) reported that while the serum lycopene in nonsmokers is comparable to that in habitual smokers, levels in smokers were lowered by 40% after smoking three regular cigarettes. This suggests a significant influence of oxidative stress, in the form of smoking, on circulating lycopene status. Likewise, supplementing the diet in a group of male nonsmokers with tomato juice (40 mg of lycopene) resulted
in a significant decrease in endogenous levels of lymphocyte DNA breakage, as measured by the COMET assay (Pool-Zobel et al., 1997).

Brady et al. (1996) indicated that lifestyle and physiological factors such as gender, smoking, alcohol consumption, and body mass index were not related to serum lycopene level in a population-based group of 400 individuals. Nonetheless, lower serum lycopene appears to be associated with older age and lower non-HDL cholesterol. Furthermore, Snowdon et al. (1996) also found a corresponding correlation between high blood lycopene level and a positive influence on the functional capacity of the elderly, such as the ability to perform self-care tasks. Higher lycopene concentration in body fat, on the other hand, was associated with a lower risk of heart attack in a group of 1,379 European men; the findings from this EURAMIC study are among the first to link lycopene to protection against heart disease (Kohlmeier et al., 1997).

Lycopene has been shown in in-vivo research to inhibit carcinogenesis in specific animal model systems (Wang et al., 1989; Nagasawa et al., 1995; Narisawa et al., 1996) as well as human cell cultures (Kim, 1995; Matsushima-Nishiwaki et al., 1995; Countryman et al., 1991; Bertram, 1993). For example, the development of spontaneous mammary tumors in SHN virgin mice was greatly reduced in the group of mice receiving lycopene-enriched food compared to the control group (Nagasawa et al., 1995). According to Mitamura et al. (1996), lycopene’s inhibitory role in mammary tumorigenesis of mice may be its ability to decrease the gene expression of transforming growth factor alpha (TGFalpha). Narisawa et al. (1996) reported the
prevention of colon carcinogenesis in rats given small doses of lycopene and lutein. Recently, lycopene was shown to act synergistically with \(\alpha\)-tocopherol to inhibit the proliferation of human prostate carcinoma cells at physiological concentration (Pastori et al., 1998). While all these studies continue to show that lycopene plays a role in preventing certain types of cancers, the mechanism of action remains unclear.

Lycopene's ability to act as an antioxidant and scavenger of free radicals that are often associated with carcinogenesis is potentially a key to the mechanism for its beneficial effects on human health (Khachik et al., 1995). As a result of having an extensive chromophore system of conjugated carbon-carbon double bonds, lycopene can accept energy from various electronically-excited species (Figure 1.12). This is attributable to its ability to quench singlet oxygen (Di Mascio et al., 1989), formed by energy transfer from a meta-stable excited photosensitizer (Krinsky, 1998). Lycopene may prevent carcinogenesis and atherogenesis by interfering passively with oxidative damage to DNA and lipoproteins (Gester, 1997; Clinton, 1998). It may also inhibit the formation of LDL cholesterol's oxidized products, which, in turn, have been suggested to participate in the early stages of coronary heart disease (Ojima et al., 1993; Diaz et al., 1997; Weisburger, 1998). Lycopene's protective effects against oxidative stress are also illustrated when human skin is irradiated with UV light - lycopene was found to be preferentially destroyed relative to \(\beta\)-carotene, suggesting either a more-active or a more-effective role (Ribayo-Mercado et al., 1995).

Despite the overwhelming evidence linking lycopene to various beneficial bioactivities, a number of inconsistencies exist in the epidemiological data regarding
Figure 1.12. Singlet-oxygen quenching by lycopene. (Adapted from Krinsky, 1998). IC: Intersystem Crossing; R and V: Rotational and Vibrational interactions with the solvent.
lycopene’s role in disease prevention. For example, Steinmetz et al. (1993) found no association between lung cancer risk and either tomatoes or three carotenoid-rich food groups. Likewise, Jarvinen et al. (1997) found that lycopene intake was not significantly related to the occurrence of breast cancer in a Finnish prospective cohort study. Differences in the oxidative environment of the lung compared to other cancer sites and the uniqueness of breast cancer carcinogenesis have been cited as key factors influencing lycopene’s effectiveness in these cases. These findings, nonetheless, suggest that further research on lycopene is needed not only to discover lycopene’s mode of action but also to understand the scope of its effectiveness.

Paradigm Shift

Over the past 25 years, the diet–health paradigm of foods being the source of essential nutrients to sustain life and growth has gradually evolved into one in which foods are also called on to deliver physiological benefits in the management or prevention of diseases. The paradigm shift is due in part to the body of epidemiological evidence associating diets rich in fruits and vegetables with the reduced risk of developing certain types of cancer and other chronic diseases. Lycopene, as discussed in this review, is among a number of compounds in fruits and vegetables that are potentially responsible for such associations. In fact, the most recent comprehensive review of 72 independent epidemiological studies revealed that intake of tomatoes and tomato-based products is inversely associated with the risk of developing cancers at several anatomic sites including the prostate gland, stomach and lung. Data were also suggestive for breast,
cervical, colorectal, esophageal, oral cavity, and pancreatic cancers (Giovannucci, 1999). Nonetheless, a direct cause-effect correlation can not be presently made regarding lycopene and reduced cancer risks without results from long-term intervention trials since epidemiological data are observational. Until such direct correlations are made, findings from human cell-culture and animal studies will continue to be relied upon to substantiate lycopene’s potential role in the observed relationship between higher intake of tomato products and lower cancer risks.

It has been suggested that as much as 20–42% of all cancer deaths in the United States are preventable by changes in dietary intake (Willett, 1995). In general, the median intake of fruits and vegetable in the U.S. falls far short of the recommendation by the National Cancer Institute (Subar et al., 1995). Fortunately, the consumption of tomatoes and tomato products is relatively high in the U.S., where the average daily dietary intake of lycopene is 3.7 mg (Forman et al., 1993) - more than three times the average intake in England, for example (Scott et al., 1996). In fact, the mean lycopene intake between 1987 and 1992 has increased among adults age 18–69 years by 5–6% (Nebeling et al., 1997).

At the very least, lycopene can be considered a nutritional indicator of good dietary habits and healthy lifestyles. Meanwhile, further research on lycopene in terms of its stability, bioavailability, and metabolism continue to be the next logical step to ultimately better understand its role in human health.
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Chapter 2

AN IMPROVED C₃₀ HPLC METHOD FOR THE DETERMINATION OF CIS-TRANS ISOMERS OF CAROTENOIDS AND PROVITAMIN A NUTRIENTS IN FRESH AND PROCESSED FRUITS AND VEGETABLES.

Updated from published manuscript:

2.1. Abstract

In this study, a reversed-phase high performance liquid chromatography (HPLC) method was developed to optimize separations of carotenoids. The polymeric C₃₀ stationary phase provides excellent resolution of all-trans carotenoids and possesses outstanding shape selectivity toward predominant geometrical isomers. Several geometrical isomers of carotenoids were found in extracts of various fresh and processed fruit and vegetables. Several cis isomers of lycopene in iodine-isomerized tomato extracts were also resolved. Increases in the quantity of geometrical isomers were observed in relation to the severity of thermal processing. Nutritional and physiological implications for the presence of these phytochemicals in the food supply are considered.
2.2. Introduction

Certain carotenoids are metabolic precursors of vitamin A and exhibit biological activity as singlet oxygen quenchers. Among dietary carotenoids, various geometric (cis and all-trans) isomers either occur naturally, or are formed during thermal processing of fruits and vegetables (Chandler and Schwartz, 1987). With provitamin A carotenoids, cis isomeric forms are less efficiently converted to vitamin A than their all-trans counterparts (Zechmeister, 1962). The measurement of geometric forms of provitamin A carotenoids is, therefore, dependent upon resolving and quantifying cis-carotenoids in foods. With respect to nutritional and other health-related aspects of carotenoid metabolism, the possibility that unique or altered physiological roles could be associated with cis versus all-trans carotenoids has not yet been rigorously addressed. Thus, the ability to accurately determine cis-trans carotenoids present in fresh and processed fruits and vegetables is a prerequisite to acquiring a better understanding of these phytonutrients in the diet. Recently a new polymeric C30 stationary phase was developed to optimize separation of carotenoids (Emenhiser et al., 1995). The new system has been shown to possess outstanding shape selectivity toward carotenoid geometric isomers.

The aim of this report is to describe an improved C30 HPLC methodology for the analysis of cis-trans carotenoids present in fresh and processed fruits and vegetables. In addition, the developed methodology is applied to monitor the chemical changes, which occur to carotenoids during thermal processing. This information is useful in assessing both nutritional and physiological implications of these phytochemicals in the diet.
2.3. Materials and Methods

Standards of all-trans β-carotene, α-carotene and lycopene were purchased from Sigma Chemical Co. (St. Louis, MO) and all-trans β-cryptoxanthin was a gift from Hoffmann-LaRoche (Nutley, NJ). All extraction and HPLC solvents (Fisher Scientific Colk Fairlawn, NJ) were certified HPLC or ACS grade. The fruits and vegetables evaluated were purchased from local markets and processed according to standard time and temperature requirements (National Canner’s Association, 1976).

2.3.1 Extraction and Saponification:

The following extraction procedure was carried out under subdued light to prevent isomerization and photodegradation. Fresh and processed carrots were diced and 10.0 g samples were homogenized in 50 mL methanol with 1.0 g CaCO₃ and 3.0 g Celite. Samples were successively extracted with a mixture of 1:1 acetone/hexane and vacuum-filtered through Whatman paper No. 1 and 42. The filtrates were combined in a separatory funnel and water was added to induce phase separation. The hexane layer was removed and brought up to volume. Triplicate 5 mL samples were dried by flushing with nitrogen.

2.3.2 Chromatography:

The reversed-phase HPLC system used in this study consisted of a Waters (Milford, MA) 2690 separation module. Separations were achieved using analytical (250 x 4.6 mm I. D.) 3μm and 5μm polymeric C₃₀ columns which were prepared at the
National Institute of Standards and Technology (Gaithersburg, MD) according to Sander et al. (1994). Guard columns packed with either C_{18} or C_{30} stationary phase were used in-line for all separations. The mobile phase was 20% methyl-t-butyl ether (MTBE) in methanol, flowing isocratically at 1.0 mL per minute. Lycopene isomer separation, on the other hand, was carried out at 1.0 mL per minute using a linear gradient of 40 to 50% MTBE in methanol for 35 minutes. Extracts were analyzed in duplicate.

### 2.3.3. Recovery Determinations:

Recovery experiments during extraction, saponification and chromatography procedures were measured. A known quantity of purified all-trans β-carotene standard was added to homogenized plant tissue. Extraction, saponification, and all chromatography procedures with and without added standard β-carotene were analyzed in duplicate. Results of recovery determinations were found to be greater than 95%. *Cis*-isomer content of samples with added standards remained the same as control extracts indicating that isomerization did not occur during the handling and chromatographic procedures.

### 2.3.4. Iodine Isomerization:

Lycopene standard or tomato extracts were solubilized in 2 mL of hexane and treated with 100 μL of 1.0 % (w/v) iodine hexane solution. Following a brief exposure to direct sunlight, the mixture was dried, resuspended in mobile phase and injected.
2.3.5. Peak Identification:

Column effluent was monitored via a Waters 996 Photodiode Array Detector at 200-800 nm with a scanning rate of 1 scan per second and 1.2 nm spectral resolution. The detector was linked to a Digital (Maynard, MA) S133 Venturis computer with Waters Millennium 2010 chromatography software (LC Version 2.15.01). Quantification of geometric isomers was achieved using a standard curve of the corresponding all-trans isomers (Sigma, St. Louis, MO) and their molar absorptivity coefficients (Britton, 1995). This method approximates the cis-isomer content, as the molar extinction coefficient values for individual lycopene isomers are not known.

Chromatographic peak identification was based on comparison to previously reported separations on polymeric C30 columns and UV-visible absorption spectral libraries. Peaks which are identified as cis-lycopene isomers have been ascertained to not be oxygen-addition products of lycopene using electrospray mass spectroscopy (Emenhiser et al., 1996; Clinton et al., 1996). The all-trans lycopene isomer peak was assigned based on retention time and co-chromatography of authentic standard.
2.4. Results and Discussion

Figure 2.1 illustrates the chromatographic separation of common carotenoids found in fruits and vegetables. The mixture consists of all-\textit{trans} standards of lutein, zeaxanthin, $\beta$-cryptoxanthin, $\alpha$-carotene and $\beta$-carotene. The peaks are identified using spectral data generated by the photodiode array (PDA) detector. The PDA capability allows for full ultraviolet and visible spectra to be obtained during analysis and is particularly useful to identify the pigments as well as their corresponding \textit{cis} isomers. Baseline resolution of the carotenoids was achieved within 35 minutes. In addition, excellent separation of lutein and zeaxanthin, two carotenoids which often co-elute during reversed-phase chromatography, is also demonstrated. Lycopene is more strongly retained on the C$_{30}$ stationary phase and does not elute within a reasonable time using the mobile phase conditions of 20\% MTBE in methanol. Analysis of lycopene requires greater concentrations of MTBE for elution or the use of gradient programming for rapid separation and analysis of these components.

Analysis of extracts of fresh carrots resulted in the chromatogram depicted in Figure 2.2A. The carotenoids shown are well separated and are typical for the natural occurrence of lutein, $\alpha$-carotene and $\beta$-carotene. These carotenoids are present in their all-\textit{trans} configuration. Although several other components were also separated and detected when monitoring at 450 nm, these components do not predominate and were not identified. Typically, carrots contain other hydrocarbon carotenoids in addition to $\alpha$ and $\beta$ carotene such as $\gamma$-carotene, $\zeta$-carotene, neurosporene, phyofluene, and phytoene (Gross, 1991). These carotenoid compounds all have similar structures as well as physical/chemical properties.
Figure 2.1. Separation of carotenoid standards by reversed-phase C$_{30}$ HPLC with photodiode array detection.

A. lutein, B. zeaxanthin, C. $\beta$-cryptoxanthin, D. $\alpha$-carotene and E. $\beta$-carotene.
Figure 2.2. Carotenoid pattern of A. raw and B. processed carrot extracts.

1. all-trans lutein, 2. 13-cis α-carotene, 3. a cis-α carotene,
4. 13'-cis-α carotene, 5. 15-cis β-carotene, 6. a cis β-carotene,
7. 13-cis β-carotene, 8. all-trans α-carotene, 9. 9-cis α-carotene,
10. all-trans β-carotene, 11. 9-cis β-carotene.
and thus would be expected to be present within these extracts.

Figure 2.2B represents a chromatogram typical of thermally processed canned carrots. The extracts contain substantial amounts of geometric isomers that were generated during the heat treatment used for canning. Such isomerization reactions induced by thermal treatments are well known and have been previously observed in processed foods (Chandler and Schwartz, 1988). The β-carotene isomers observed consisted of the 15-cis, 13-cis, all-trans and 9-cis components which were identified in the processed carrot extract. Many more isomers of α-carotene were detected, as the structure of this carotenoid is asymmetrical in contrast to β-carotene, which has a symmetrical structure. The 13-cis, 13'-cis, all-trans and 9-cis isomers of α-carotene are well resolved (Fig. 2.2B). The 9'-cis isomer of α-carotene is not shown because this compound co-elutes with all-trans β-carotene. Resolution of this geometric isomer from other α-carotene isomers can be achieved using the C₃₀ system (Emenhiser et al., 1996); however, if all-trans β-carotene is present, detection of this compound is difficult. Cis forms of lutein were also observed to form during the thermal treatment. However, since the mobile phase used was optimized to separate the hydrocarbon carotenoids, elution of the xanthophylls occurred within the early eluting peaks, which prevented their identification.

The chromatograms shown in Figure 2.2 are illustrative of several important considerations regarding the application of the C₃₀ column to carotenoid separations. The chromatographic profile of carotenoids found in fresh carrots is quite simple relative to those found in processed samples. For applications where the carotenoid
composition is complex and geometrical isomers are to be analyzed, the high degree of selectivity for these compounds on the C\textsubscript{30} stationary phase may complicate identification due to co-elution problems. Employing gradient elution schemes may provide a solution when quantification of the geometric isomers in complex mixtures is necessary. Applications do exist where separations of the geometric forms are not warranted. However, for carotenoid separations where this information is needed, the C\textsubscript{30} system exhibits excellent resolving capabilities for the geometric forms and better overall performance for this analysis relative to existing reversed-phase columns. Figure 2.3 illustrates the chain length of the C\textsubscript{30} system relative to that of the shorter C\textsubscript{18} counterpart. The extended chain length appears to allow for enhanced interaction between carotenoids and the stationary phase (Sander \textit{et al.}, 1994).

C\textit{i}s isomers of the provitamin A carotenoids (i.e. $\beta$-cryptoxanthin, $\alpha$-carotene and $\beta$-carotene) have provitamin A activities which are approximately 50% or less than the corresponding all-\textit{trans} carotenoids (Zechmeister, 1962; Sweeney and Marsh, 1973). Therefore, in order to quantitate the provitamin A content of foods and the effects of processing on their nutritional value with respect to vitamin A, the various isomeric forms of these carotenoids present in both the fresh and processed state must be accurately determined. The chromatographic methodology described in this study will assist in providing more accurate information of vitamin A content for nutritional tables and databases. In addition, given the current interest of these antioxidants in health and disease, understanding the role and presence of carotenoid isomers in the diet may impact studies involving these compounds with specific physiological and
Figure 2.3. Comparison of molecular length to reverse phase stationary phase thickness as determined by small angle neutron scattering. Adapted from Sander et al., 1994.
biological activities (Levin and Mokady, 1994; Wang et al., 1994; Gaziano et al., 1995).

Although a large percentage of lycopene found in biological tissues such as in human sera exists in the cis configuration, it does not appear that the cis forms of lycopene are present in processed foods or processed tomato products (Figure 2.4A). Figure 2.4B illustrates the separation and distribution of lycopene isomers obtained from an iodine-isomerized mixture. The chromatogram depicts the resolving capabilities of the C30 stationary phase to separate cis-trans isomers of lycopene. Other reversed-phase separations of lycopene will generally not resolve cis isomers and typical chromatograms will commonly show the cis forms eluting as a shoulder present with the all trans peak.

The geometric configurations of all the isomers shown on the C30 separation have not yet been identified and will require further work, isolating the pure isomers and obtaining nuclear magnetic resonance (NMR) spectra. However, all of the separated lycopene isomers shown in Figure 2.4B (retention time > 10 minutes) exhibited similar ultra-violet visible absorption spectral characteristics for lycopene. In addition, mass spectral determinations for the isolated lycopene fractions all confirm molecular weights of 536 a.m.u. providing additional evidence for their identity as lycopenes (Clinton et al., 1996). While other carotenoids (i.e. β-carotene) readily isomerize during the processing of food products, lycopene seems relatively stable towards thermally-induced isomerization reactions in tomato products. Thus, the presence of the cis lycopenes measured in biological tissues cannot be attributed to the consumption of cis lycopenes from tomato products in the diet.
Figure 2.4. Separation of lycopene isomers in A. processed and B. iodine-isomerized tomato extracts.
2.5. References


2.6 Acknowledgements:

The authors thank Dr. Lane Sander of the National Institute of Standards and Technology, Gaithersburg, MD for providing the polymeric C$_{30}$ columns used in this project.

Assistance provided by members of the Carl E. Haas Endowment Chair laboratory group is also acknowledged with gratitude.
Chapter 3

LYCOPENE STABILITY DURING FOOD PROCESSING

Updated from published manuscript:


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3.1. Abstract:

Accumulating epidemiological evidence continues to show that lycopene, found in tomatoes, grapefruits and watermelons, is associated with a reduced risk of developing certain chronic diseases and cancers. With respect to lycopene in tomato products, the effect of thermal processing on its stability has not yet been rigorously addressed. This chapter assesses the effect of several different heat treatments on lycopene's isomeric distribution in a variety of tomato products, as well as in organic solvent mixtures containing all-trans lycopene. Experimental results indicate that in contrast to β-carotene, lycopene remained relatively resistant to heat-induced geometrical conversion during typical food processing of tomatoes and related products. The presence of fat, the change in percent solids and the severity of heat treatment were not contributing factors in the formation of lycopene isomers in tomato products, except at extreme conditions not regularly employed in the food industry or during food preparation. Lycopene in organic solvent, however, isomerized readily as a function of time even in the absence of light and in the presence of antioxidants. These findings suggest that while lycopene is stable in the tomato matrix, sample-handling techniques should be carefully evaluated to minimize the formation of lycopene cis isomers in organic solutions.
3.2. Introduction

In recent years, there has been considerable epidemiological evidence that the carotenoid lycopene may reduce the risk of developing cervical, colon, prostate, rectal and stomach cancers (Batieha et al., 1993; Block et al., 1992; Giovannucci et al., 1995; Snowdon, 1996). For example, Giovannucci and colleagues (1995) concluded that for a cohort of 47,894 male subjects, consumption of fresh tomatoes, tomato sauce and pizza, which account for the bulk of lycopene intake is significantly related to a lower incidence of prostate cancer. These findings have prompted considerable interest in understanding the role of lycopene in the diet and its stability in foods.

In fresh tomatoes as well as in other fruit and vegetables, lycopene has been found to occur predominantly in the all-\textit{trans} geometrical configuration (Stahl and Sies, 1992; Khachik et al., 1992a; Wilberg and Rodriguez-Amaya, 1995; Emenhiser et al., 1995). In contrast, \textit{cis} lycopene isomers have been detected in plasma and tissue samples at significant levels (Khachik et al., 1992b; Stahl et al., 1993; Clinton et al., 1996). Clinton and coworkers (1996) reported that lycopene in serum and prostate tissues is predominantly in the \textit{cis} isomeric form, comprising about 58-88% of the total lycopene content.

To date, isomerization of fruits and vegetable carotenoids has been attributed to thermal treatment during food processing and preparation (Chandler and Schwartz, 1988; Quackenbush, 1987; Sweeney and Marsh, 1971; Lessin et al., 1997). For example, Lessin \textit{et al} (1997) reported that $\beta$-carotene \textit{cis} isomer content increases from 12.9% in fresh tomatoes to 31.2% in canned tomatoes. With respect to lycopene, Stahl
and Sies (1992) reported that heating of tomato juice not only increased its cis-lycopene isomer content but also lead to an improvement in uptake of overall lycopene in humans. Likewise, Schierle and coworkers (1997) found that bench-top preparation of a spaghetti sauce from canned tomatoes increased the level of lycopene cis isomers. Another recent study by Gartner et al. (1997) reiterates the notion that tomato paste, as a processed product, has more bioavailable lycopene than fresh tomatoes when both are consumed in conjunction with corn oil.

One of the difficulties in assessing isomerization reactions of lycopene in foods is the ability to separate the complex mixture of the cis-trans isomers of this carotenoid. Recently, we have been employing a specialized C<sub>30</sub> high performance liquid chromatography (HPLC) column, which exhibits excellent resolution of over ten different lycopene isomers (Emenhiser et al., 1995). In this report, we apply this HPLC methodology to monitor the extent of lycopene isomerization during typical thermal processing treatments of tomatoes and tomato products. A better understanding of lycopene’s thermal stability in tomato products may be useful in assessing both its nutritional and physiological implications in the diet.
3.3 Materials and Methods:

Standards of all-trans β-carotene, lycopene and butylated hydroxytoluene were purchased from Sigma Chemical Co. (St. Louis, MO). All extraction and HPLC solvents (Fisher Scientific Co. Fairlawn, NJ) were certified HPLC or ACS grade. The fruits and vegetables evaluated were purchased from local markets and heat treated according to standardized industrial food processing requirements (National Canner’s Association, 1976).

3.3.1 Extraction and Saponification:

The following extraction procedure was carried out under subdued light to prevent isomerization and photodegradation. Fresh and processed tomatoes were diced and 10.0 g samples were homogenized in 50 mL methanol with 1.0 g CaCO₃ and 3.0 g Celite. Samples were successively extracted with a mixture of 1:1 acetone/hexane and vacuum-filtered through Whatman paper No. 1 and 42. The filtrates were combined in a separatory funnel and water was added to induce phase separation. The hexane layer was removed and brought up to volume. Extracts of samples containing lipids were saponified with 30% KOH for 60 minutes. Triplicate 3 mL samples were dried by flushing with nitrogen and extracts were analyzed in duplicate by HPLC.

3.3.2 Lycopene Isomerization in Organic Solvent:

Triplicates of 2 mL samples containing all-trans lycopene in 1:1 methanol and methyl-tert-butyl ether (MTBE) were incubated in the dark at a. 4°C, b. 27°C, and 27°C
with 0.02% BHT added. Samples were analyzed at 0, 60, 120 and 180 minutes in cubation time by HPLC.

3.3.3. HPLC Analysis:

The reverse-phase HPLC system used in this study consisted of a Waters (Milford, MA) 2690 separation module. Separations were achieved using analytical (250 x 4.6 mm I. D.) 3μm polymeric C₃₀ columns which were prepared at the National Institute of Standards and Technology, Gaithersburg, MD by Dr. Lane Sander as described by Sander et al., 1994. Guard columns packed with C₃₀ stationary phase were used in-line for all separations (YMC, Inc., Wilmington, NC). Lycopene isomer separation was carried out at 1.0 mL per minute using a linear gradient of 40 to 50% MTBE in methanol for 35 minutes.

3.3.4. Recovery Determinations:

Results of recovery determinations during extraction, saponification and chromatography procedures were found to be greater than 95%. Cis-isomer content of samples with added standards remained the same as control extracts, indicating that isomerization did not occur during the handling and chromatographic procedures.

3.3.5. Peak Identification:

Column effluent was monitored via a Waters 996 Photodiode Array Detector at 200-800 nm with a scanning rate of 2 scan/second and 1.2 nm spectral resolution. The detector was linked to a Digital (Maynard, MA) S133 Venturis computer with Waters 94
Millennium 2010 chromatography software (LC Version 2.15.01). Quantification of lycopene geometric isomers was achieved using a standard curve of the all-\textit{trans} isomer (Sigma, St. Louis, MO) and its molar absorptivity coefficient (Britton, 1995). This method approximates the \textit{cis}-isomer content, as the molar extinction coefficient values for individual isomers are not known.

Chromatographic peak identification was based on comparison to previously reported separations on polymeric C$_{30}$ columns and UV-visible absorption spectral libraries. Peaks which are identified as \textit{cis}-lycopene isomers have been ascertained using electrospray mass spectroscopy (Clinton \textit{et al.}, 1996) to have the same molecular weight as all-\textit{trans} lycopene and thus are not oxygen-addition products. The identities of all-\textit{trans} isomer peaks were assigned based on retention time and co-chromatography of authentic standards.
3.4. Results:

Reversed-phase chromatography of a typical fresh tomato extract is depicted in Figure 3.1. In red tomato, the all-\textit{trans} lycopene isomer predominates, comprising 95.4% of the total lycopene content. Analysis of commercial tomato products such as vegetable juice, tomato juice, tomato sauce, tomato soup, tomato paste, pizza sauce, spray-dried and drum-dried tomato powders and sun-dried tomatoes in oil indicates that of all the samples tested none had a total \textit{cis} isomer content greater than 10.1% (Table 3.1). Although all of these products were processed under different thermal conditions, their \textit{cis} isomer contents do not reflect the relative amount of heat treatment that the products are believed to have received. For example, the hot-filled tomato juice (82°C, 30 sec) and tomato paste (104°C, 50 min) samples did not differ significantly in terms of \textit{cis} isomer levels despite the more rigorous thermal treatment of the latter.

A variety of tomato products were produced at the Ohio State University Food Industry Center to further investigate the formation of \textit{cis} isomers as a result of thermal processing. Unlike the commercial tomato products purchased from local markets, the products made at the Food Industry Center allow for overall control and monitoring of the tomato preparation and processing steps (Figure 3.2). The lycopene content and isomeric distribution are reported in Table 3.2 for these products. The results suggest that lye peeling (18% NaOH treatment) of tomato at 82°C for 15 seconds does not induce any \textit{cis} isomer formation. This is consistent with previous observations that non-oxygenated carotenoids such as lycopene and other carotenes are stable against basic conditions such as during saponification using 30% NaOH (Emenhiser \textit{et al}.,
Figure 3.1. Representative C$_{30}$ HPLC chromatographic separation of lycopene extracted from fresh tomatoes using a 60:40 to 50:50 (v/v) methanolic MTBE mobile phase gradient.
Figure 3.2. Schematic of thermal processing steps and conditions for various tomato products produced at The Ohio State University Food Industry Center.
## Commercial Products

<table>
<thead>
<tr>
<th>Type</th>
<th>Lycopene content (mg/100g (wet wt.))</th>
<th>Number of analyses</th>
<th>All-trans isomer percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tomato juice</td>
<td>7.83 ± 1.33</td>
<td>6 x 6</td>
<td>93.8 ± 2.2</td>
</tr>
<tr>
<td>Vegetable juice</td>
<td>7.28 ± 1.17</td>
<td>7 x 6</td>
<td>95.5 ± 1.9</td>
</tr>
<tr>
<td>Tomato soup (condensed)</td>
<td>7.99 ± 1.23</td>
<td>6 x 6</td>
<td>93.9 ± 2.3</td>
</tr>
<tr>
<td>Salsa</td>
<td>9.08 ± 0.76</td>
<td>6 x 4</td>
<td>94.4 ± 2.8</td>
</tr>
<tr>
<td>Whole tomatoes (canned)</td>
<td>11.21 ± 1.06</td>
<td>1 x 6</td>
<td>97.5 ± 1.2</td>
</tr>
<tr>
<td>Ketchup</td>
<td>13.44 ± 2.46</td>
<td>2 x 6</td>
<td>93.8 ± 2.8</td>
</tr>
<tr>
<td>Spaghetti sauce</td>
<td>17.12 ± 3.94</td>
<td>5 x 6</td>
<td>91.1 ± 3.4</td>
</tr>
<tr>
<td>Pizza sauce (canned)</td>
<td>12.71 ± 1.69</td>
<td>2 x 6</td>
<td>89.9 ± 3.5</td>
</tr>
<tr>
<td>Sauce from pizza</td>
<td>32.89 ± 3.11</td>
<td>1 x 6</td>
<td>95.4 ± 3.0</td>
</tr>
<tr>
<td>Tomato paste</td>
<td>30.07 ± 1.73</td>
<td>2 x 6</td>
<td>93.2 ± 2.4</td>
</tr>
<tr>
<td>Sun-dried tomato in oil</td>
<td>46.50 ± 4.51</td>
<td>1 x 6</td>
<td>92.3 ± 3.2</td>
</tr>
<tr>
<td>Spray-dried tomato powder</td>
<td>126.49 ± 5.64</td>
<td>1 x 6</td>
<td>94.7 ± 2.7</td>
</tr>
<tr>
<td>Drum-dried tomato powder</td>
<td>112.63 ± 7.57</td>
<td>1 x 6</td>
<td>93.8 ± 2.4</td>
</tr>
<tr>
<td>Sigma lycopene standard</td>
<td>N/A</td>
<td>3 x 6</td>
<td>93.4 ± 3.6</td>
</tr>
</tbody>
</table>

Table 3.1. Lycopene content and relative abundance of lycopene all-\textit{trans} isomers in various commercial tomato products.
<table>
<thead>
<tr>
<th>Type</th>
<th>Processing treatment</th>
<th>Lycopene content mg/100g (dry wt.)</th>
<th>Lycopene cis isomer %</th>
<th>β-carotene cis isomer %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tomatoes</td>
<td>None</td>
<td>152.98</td>
<td>4.16</td>
<td>21.77</td>
</tr>
<tr>
<td>Tomatoes</td>
<td>Lye-peeled</td>
<td>149.89</td>
<td>5.37</td>
<td>23.83</td>
</tr>
<tr>
<td>Tomato juice</td>
<td>Hot-break</td>
<td>161.23</td>
<td>5.98</td>
<td>57.55</td>
</tr>
<tr>
<td>Tomato juice</td>
<td>Retorted</td>
<td>180.10</td>
<td>3.56</td>
<td>78.28</td>
</tr>
<tr>
<td>Whole tomatoes</td>
<td>Retorted</td>
<td>183.49</td>
<td>3.67</td>
<td>62.03</td>
</tr>
<tr>
<td>Tomato paste</td>
<td>Concentrated</td>
<td>174.79</td>
<td>5.07</td>
<td>57.82</td>
</tr>
<tr>
<td>Tomato paste</td>
<td>Retorted</td>
<td>189.26</td>
<td>4.07</td>
<td>85.85</td>
</tr>
<tr>
<td>Tomato soup</td>
<td>Retorted</td>
<td>139.56</td>
<td>4.34</td>
<td>55.57</td>
</tr>
<tr>
<td>Tomato sauce</td>
<td>Retorted</td>
<td>84.33</td>
<td>5.13</td>
<td>56.14</td>
</tr>
<tr>
<td>Yellow tomatoes</td>
<td>None</td>
<td>N/A</td>
<td>N/A</td>
<td>11.08</td>
</tr>
<tr>
<td>Yellow tomatoes</td>
<td>Cooked</td>
<td>N/A</td>
<td>N/A</td>
<td>26.56</td>
</tr>
<tr>
<td>Tomato in oil</td>
<td>Heated</td>
<td>N/A</td>
<td>10.67</td>
<td>N/A</td>
</tr>
</tbody>
</table>

Table 3.2. Lycopene content and relative abundance of lycopene isomers in various thermally-processed tomato products prepared at the Food Industry Center.

Tomato soup and sauce lycopene values are adjusted to account for oil content. Analysis are performed in duplicate for three representative samples. The reported values are the averages of the three means.
The lycopene level is slightly lower for peeled tomato as the removed peel material is known to have higher lycopene content (Sharma and Le Maguer, 1996). Tomatoes, which were macerated prior to juicing, have essentially the same lycopene content and isomer distribution as the resulting tomato juice. Thermal treatment in a holding tube at 82°C for 15 seconds to pasteurize the juice did not induce isomer formation. Likewise, as the juice and whole peeled tomatoes in juice are canned and heat treated at 104°C for 50 minutes, their lycopene isomeric distributions did not change either.

Further thermal treatment of the remaining pasteurized juice at 85-95°C for 3 hours to concentrate it into paste did not affect either the formation of lycopene isomers or their relative abundance. Even the tomato soup and sauce, which were made from paste and heat processed at 104°C for 50 minutes, did not exhibit significant increases in cis lycopene isomer levels. The presence of olive oil at 5% and 15% in the tomato sauce did not affect the isomer content compared to the tomato soup with only 2% fat (Table 3.3). The use of glass jars as containers to evaluate light exposure and inertness of contact surfaces did not perceptibly impact the formation of cis isomer in all processed products tested. Although lycopene did not isomerize under any of the processing conditions above, appreciable levels of β-carotene cis isomers did form within the tomato tissue during all of these processing treatments (Table 3.2).

Formation of lycopene cis isomers was successfully induced by excessive thermal exposure, such as heating chopped tomato in olive oil at ~200°C for 45 minutes. Similarly, high temperature heating of a thin film of pureed tomato at 200°C for a few
<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Description</th>
<th>Amount</th>
<th>Unit</th>
<th>Approximate Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tomato Paste</td>
<td>9.8 Brix</td>
<td>8950.00</td>
<td>gram</td>
<td>89.07</td>
</tr>
<tr>
<td>Vegetable Oil</td>
<td>Olive</td>
<td>500.00</td>
<td>gram</td>
<td>4.98</td>
</tr>
<tr>
<td>Sugar</td>
<td>Granulated</td>
<td>250.00</td>
<td>gram</td>
<td>2.49</td>
</tr>
<tr>
<td>Salt</td>
<td>Granulated</td>
<td>160.90</td>
<td>gram</td>
<td>1.60</td>
</tr>
<tr>
<td>Vinegar</td>
<td>Distilled</td>
<td>49.00</td>
<td>ml</td>
<td>0.49</td>
</tr>
<tr>
<td>Starch</td>
<td>Corn</td>
<td>44.00</td>
<td>gram</td>
<td>0.44</td>
</tr>
<tr>
<td>Paprika</td>
<td>Powder</td>
<td>16.90</td>
<td>gram</td>
<td>0.17</td>
</tr>
<tr>
<td>Onion</td>
<td>Dehydrated</td>
<td>8.00</td>
<td>gram</td>
<td>0.08</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td></td>
<td>~10,000</td>
<td>gram</td>
<td>100 %</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Description</th>
<th>Amount</th>
<th>Unit</th>
<th>Approximate Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tomato Paste</td>
<td>9.8 Brix</td>
<td>8000.00</td>
<td>gram</td>
<td>79.61</td>
</tr>
<tr>
<td>Vegetable Oil</td>
<td>Olive</td>
<td>1500.00</td>
<td>gram</td>
<td>14.93</td>
</tr>
<tr>
<td>Sugar</td>
<td>Granulated</td>
<td>250.00</td>
<td>gram</td>
<td>2.49</td>
</tr>
<tr>
<td>Salt</td>
<td>Granulated</td>
<td>160.90</td>
<td>gram</td>
<td>1.60</td>
</tr>
<tr>
<td>Vinegar</td>
<td>Distilled</td>
<td>49.00</td>
<td>ml</td>
<td>0.49</td>
</tr>
<tr>
<td>Starch</td>
<td>Corn</td>
<td>44.00</td>
<td>gram</td>
<td>0.44</td>
</tr>
<tr>
<td>Paprika</td>
<td>Powder</td>
<td>16.90</td>
<td>gram</td>
<td>0.17</td>
</tr>
<tr>
<td>Onion</td>
<td>Dehydrated</td>
<td>8.00</td>
<td>gram</td>
<td>0.08</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td></td>
<td>~10,000</td>
<td>gram</td>
<td>100 %</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Description</th>
<th>Amount</th>
<th>Unit</th>
<th>Approximate Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tomato Paste</td>
<td>9.8 Brix</td>
<td>8850.00</td>
<td>gram</td>
<td>94.54</td>
</tr>
<tr>
<td>Vegetable Oil</td>
<td>Olive</td>
<td>190.00</td>
<td>gram</td>
<td>2.03</td>
</tr>
<tr>
<td>Sugar</td>
<td>Granulated</td>
<td>119.00</td>
<td>gram</td>
<td>1.27</td>
</tr>
<tr>
<td>Salt</td>
<td>Granulated</td>
<td>139.00</td>
<td>gram</td>
<td>1.48</td>
</tr>
<tr>
<td>Starch</td>
<td>Corn</td>
<td>49.75</td>
<td>gram</td>
<td>0.53</td>
</tr>
<tr>
<td>Onion</td>
<td>Dehydrated</td>
<td>29.00</td>
<td>gram</td>
<td>0.31</td>
</tr>
<tr>
<td>Pepper</td>
<td>Ground</td>
<td>4.00</td>
<td>gram</td>
<td>0.04</td>
</tr>
<tr>
<td>Cinnamon</td>
<td>Powder</td>
<td>0.62</td>
<td>gram</td>
<td>0.01</td>
</tr>
<tr>
<td>Mace</td>
<td>Powder</td>
<td>0.31</td>
<td>gram</td>
<td>0.00</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td></td>
<td>~10,000</td>
<td>gram</td>
<td>100 %</td>
</tr>
</tbody>
</table>

Table 3.3. Formulations of tomato products produced in this study.
seconds to rapidly remove moisture, increased the level of cis isomers from 4.2 to 19.1%. However, commercially available drum-dried tomato flakes had a lycopene cis isomer level of 6.25%.

In organic solvent, lycopene isomerized readily and the amount of cis isomers increased as a function of time. Solutions of lycopene incubated at 27°C in the absence of light and in organic solvents resulted in a cis isomer content >50% after 3 hours of incubation compared to 4.5% in the initial solution (Figure 3.3). A lower incubation temperature of 4°C did slow the formation of isomers. Similarly, the addition of butylated hydroxytoluene (BHT) as an antioxidant also reduced the rate of lycopene isomerization. It is important to note that the changes in incubation temperature and/or the presence of BHT did not alter the final equilibrium distribution of lycopene isomers.
Figure 3.3. Relative abundance of all-trans lycopene and its cis isomers in organic solvents.

A. Starting material (0 min at 27°C)
B. After incubation for 180 min at 27°C with BHT
C. After incubation for 180 min at 27°C without BHT.
3.5. **Discussion:**

Previous reports have demonstrated isomerization reactions of carotenoids during thermal treatment and food processing of fruits and vegetables (Chandler and Schwartz, 1988; Quackenbush, 1987; Sweeney and Marsh, 1971; Lessin et al., 1997). In this report, the carotenoid lycopene did not exhibit similar susceptibility to isomerization. Heating of tomatoes and the presence of lipids together may have improved the bioavailability of lycopene, as reported by Stahl and Sies (1992) and Gartner et al. (1997), but these factors were shown to not have an influence on the formation of lycopene geometrical isomers. Parker (1988) suggests that the improvement in bioavailability may be a result of the destabilization of protein-carotenoid complexes or the dissolution and dispersion of crystalline lycopene aggregates.

According to our results, heat and shear during typical industrial food processing operations did not initiate lycopene isomerization. The large difference between levels of cis lycopene isomers in tomato products or other processed foods and organic solvent mixtures indicates that thermal treatments did not free lycopene from the tomato matrix into solution to undergo geometrical conversion. Furthermore, the fact that β-carotene readily isomerizes during the processing of tomato products while lycopene remains relatively stable suggests that these carotenones have different bond energies and kinetics for isomerization reactions (Henry et al., 1998). Since the rate of isomer formation was reduced in the presence of the antioxidant BHT, geometric conversion of lycopene may occur via the free radical formation pathway suggested by Gao et al. (1996).

From this study, we conclude that thermal treatment of tomato products during usual food preparation or commercial production processes does not appear to result in a
significant shift in the distribution of cis-lycopene isomers. These findings are in contrast to β-carotene, which was observed to readily isomerize during typical processing of tomatoes. Further studies are in progress to investigate whether the presence of various cis lycopene geometrical isomers in human serum and biological tissues have dietary origin.
3.6. References:


3.7 Acknowledgements:

The authors thank Dr. Winston Bash and Gary Wenneker of the Food Industries Center at The Ohio State University for their technical support.

Assistance provided by members of the Carl E. Haas Endowment Chair laboratory group during the tomato processing trials is also acknowledged with gratitude.


Chapter 4

THERMAL ISOMERIZATION OF CAROTENOID IN DIFFERENT TOMATO VARIETIES


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Ohio Agricultural Research and Development Center
1680 Madison Avenue, Wooster, Ohio 44691
Abstract

The thermal stability of predominant tomato carotenoids is reported for five mutant varieties with distinctively different carotenoid distribution. In these varieties, the all-trans form of lycopene, its poly-cis geometrical isomer, δ-carotene, and γ-carotene remain stable to heat treatment while significant amounts of all-trans β-carotene and lutein were converted to the cis configurations. The presence of oil did not affect the thermal stability of any carotenoids being evaluated. Examination of samples by electron microscopy indicated that heat treatment imparts changes to the physical ultrastructure of the tomato tissue such as cell wall and organelle deformation.

The results indicate that common occurrences during tomato processing such as genotypical differences in overall carotenoid composition, the presence of oil and physical changes to tomato tissues did not appear to influence the thermal stability of lycopene as well as δ-carotene, γ carotene and prolycopene. β-carotene and lutein, however, readily isomerized as a result of thermal treatments. The observed differences in these carotenoids' relative susceptibility to thermally-induced isomerization reactions might be attributable to their differences in physical state and cellular localization. Thus, while thermal processing reportedly alters the bioavailability of carotenoids, it does alter the geometrical configuration of only some carotenoids and not others. These findings are important considerations in our overall effort to better understand carotenoid metabolism and the physical chemistry of lycopene in vitro.
4.2. Introduction

In recent years, dietary carotenoids such as lycopene and β-carotene have emerged as potentially beneficial phytochemicals in light of epidemiological research findings which link the consumption of food products rich in carotenoids to the reduction in risks of developing certain types of cancer (Block et al., 1992; van Poppel, 1993; Steinmetz and Potter, 1996; Giovannucci, 1999). Among dietary carotenoids, various geometrical isomers either occur naturally, or are formed during thermal processing of fruits and vegetables and food preparation (Sweeney and Marsh, 1971; Chandler and Schwartz, 1988, Lessin et al., 1997). As the various isomeric forms of dietary carotenoids may possess different biological properties, an understanding of the various factors affecting the formation of these carotenoids geometrical isomers prior to consumption and following ingestion may provide insights into the potentially important association between structure and function. It is well-known in the case of provitamin A carotenoids such as α-carotene and β-carotene, for example, that the cis isomeric forms are less efficiently converted to vitamin A than the all-trans counterparts (Zechmeister, 1962).

For the carotenoid lycopene, which has no provitamin A activity, the relative abundance of lycopene molecules in the linear all-trans configuration compared to those in the bent cis forms appears to be variable as a function of medium. Intriguing bioavailability and human tissue deposition patterns in terms of relative concentration and isomeric distribution have been reported by several investigators (Stahl and Sies, 1992; Clinton et al., 1996; Gartner et al., 1997) for lycopene, the predominant
carotenoid in human serum, tissues, and in the diet. Clinton et al. (1996) observed that the ratio of lycopene cis-trans geometrical isomers in biological fluids such as plasma and in tissues such as prostate differs from those of fresh tomatoes. It has previously been assumed that the higher percentage of lycopene cis isomer in human biological samples is due in part to consumption of heat treated tomato products containing cis isomers of lycopene (Schierle et al., 1997; van het Hof et al., 1998). Our laboratory has recently established, however, that in tomato products of various moisture content, fat content, and container type, lycopene, unlike β-carotene, is remarkably stable to isomerization reactions under typical industrial thermal processing conditions (Nguyen and Schwartz, 1998). While a number of possible explanations exist for the observed difference in thermal stability between lycopene and β-carotene, we have designed the following experiments to focus on two possibilities, differences in the cellular localization of the carotenoids and secondly the presence or absence of β-ionone rings.

The purpose of this study, therefore, is to investigate the difference in isomerization reactions of lycopene and β-carotene in the context of structural distinction and cellular specificity. We take advantage of the fact that among the many cultivars of tomatoes, Lycopersicon esculentum, there are distinct differences in the distribution of chromoplastid carotenoid pigments including lycopene and β-carotene, which give rise to the yellow, orange and red color of mature fruits. Five mutant tomato varieties with different carotenoid composition and color will be used. The unique carotenoid composition of these mutant varieties provides an excellent opportunity to study the thermal behavior of lycopene and β-carotene. Likewise, a better
understanding of the isomerization stability of δ-carotene and γ-carotene, which are structural intermediates of lycopene and β-carotene, as well as lutein and prolycopene, which are also structurally-related to the major tomato carotenoids being studied, may provide further insights into possible mechanistic details of cis-trans isomerization reactions in tomatoes.

Transmission electron microscopy will be employed to study the ultrastructure of tomato chromoplasts and to monitor any heat-induced physical alteration of these carotenoid-bearing organelle structures. The electron micrographic information might be useful in providing a physical explanation for the different susceptibility of tomato carotenoids to food processing-induced isomerization reactions. Ultimately, this information together with the chromatographic determinations of isomer distribution in the various mutant tomato varieties before and after thermal treatment will be used to account for lycopene's stability during food processing and help to identify the main factors affecting its geometrical isomer distribution in the diet.
4.3. **Materials and Methods:**

Standards of all-\textit{trans} \(\beta\)-carotene and lycopene were purchased from Sigma Chemical Co. (St. Louis, MO). All extraction and HPLC solvents (Fisher Scientific, Fairlawn, NJ) were certified HPLC or ACS grade. Tomatoes were purchased from local markets and processed according to standard time and temperature requirements (National Canner's Association, 1976) in the Food Industries Center at Ohio State University. Samples were collected throughout the production process. The collected samples and finished products were analyzed via HPLC immediately upon collection and these results had been reported (Nguyen and Schwartz, 1998). HPLC data for finished products which were stored at room temperature in the dark and analyzed after an 18-month period are reported in this study.

Specific tomato varieties with different color and carotenoid composition were also used: Crimson-type red tomatoes (RED), high \(\beta\)-carotene (BET) and high \(\delta\)-carotene (DEL), tangerine-type tomatoes (PRO), and yellow tomatoes (LOW) with reduced carotenoid levels. These tomatoes were sectioned and heated for 30 minutes at 100\(^\circ\)C in either distilled water or an 80:20 water/olive oil mixture. Selected samples were then examined via transmission electron microscopy.

4.3.1. **Extraction and Saponification:**

The following extraction procedure was carried out under subdued light to prevent isomerization and photodegradation as previously described (Nguyen and Schwartz, 1998). Fresh and processed tomatoes were diced and 10.0 g samples were
homogenized in 50 mL methanol with 1.0 g CaCO₃ and 3.0 g Celite. Samples were successively extracted with a mixture of 1:1 acetone/hexane and vacuum-filtered through Whatman paper No. 1 and 42. The filtrates were combined in a separatory funnel and water was added to induce phase separation. The hexane layer was removed and brought up to volume. Triplicate 5 mL samples were dried by flushing with nitrogen. Extracts were analyzed in duplicate. Extracts of samples containing lipids were saponified with 30% KOH for 60 minutes before aliquots of the filtrate were dried.

4.3.2. Chromatography:

The reversed-phase HPLC system used in this study consisted of a Waters (Milford, MA) 2690 separation module. Separations were achieved using analytical (250 x 4.6 mm I. D.) 3µm polymeric C₃₀ columns which were prepared at the National Institute of Standards and Technology (Gaithersburg, MD) according to Sander et al., (1994). Guard columns packed with C₃₀ stationary phase were used in-line for all separations. Lycopene isomer separation was carried out as reported earlier (Schwartz and Nguyen, 1998) at 1.0 mL per minute using a linear gradient of 40 to 50% methyl-ᵣ-butil ether (MTBE) in methanol for 35 minutes. Total carotenoid separation was carried out at 1.0 mL per minute using a multi-step linear gradient of 15 to 50% MTBE in methanol for 55 minutes.
4.3.3. Recovery Determinations:

Results of recovery determinations during extraction, saponification and chromatography procedures were found to be greater than 95%. *Cis-*isomer content of samples with added all-\textit{trans} carotenoid standards remained the same as control extracts, indicating that artifactual isomerization did not occur during the handling of these samples and subsequent chromatographic procedures.

4.3.4. Iodine Isomerization:

\(\beta\)-Carotene and lycopene standards along with purified \(\delta\)-carotene, \(\gamma\)-carotene, and lutein extracts were solubilized in 2 mL of hexane and treated with 10 \(\mu\)L of a 1.0% iodine in hexane solution. Following brief exposure to direct sunlight, the mixture was dried, resuspended in mobile phase and injected. The resulting separations were used to tentatively identified *cis* isomers of the corresponding carotenoids being evaluated.

4.3.5. Peak Identification:

Column effluent was monitored via a Waters 996 Photodiode Array Detector at 200-800 nm with a scanning rate of 2 scans per second and 1.2 nm spectral resolution. The detector was linked to a Digital (Maynard, MA) S133 Venturis computer with Waters Millennium 2010 chromatography software (LC Version 2.15.01). Quantification of geometric isomers of carotenoids was achieved using a standard curve of the corresponding all-\textit{trans} isomers and their molar absorptivity coefficients (Britton, 1995). This method approximates the *cis*-isomer content, as the molar extinction
coefficient values for individual isomers are not known. In the case of the poly-cis lycopene isomer in Tangerine tomatoes, the molar absorptivity is known.

Chromatographic peak identification was based on comparison to previously reported separations on polymeric C_{30} columns and UV-visible absorption spectral libraries. Peaks which were identified as cis-lycopene isomers had been ascertained using electrospray mass spectroscopy (van Breemen, 1996, Emenhiser et al., 1996, Clinton et al., 1996) to have the same molecular weight as all-trans lycopene and thus are not oxygen-addition products. The identities of all-trans isomer peaks were assigned based on retention time and co-chromatography of authentic standards as well as by comparing the observed maximum absorptivity in two separate solvent systems against published values. Tentative identifications are made for the remaining carotenoids cis isomers by comparing their retention time and spectrum to the iodine photomutation mixture as well as by the appearance of “cis-peaks” approximating 140 nm downfield from the $\lambda_{\text{max}}$.

4.3.6. Transmission Electron Microscopy of Tomato Tissues:

Selected samples of fresh and processed tomato tissues were sectioned and portions were examined by light microscope. Small pieces of tissue from below the epidermis were obtained and fixed in 5% glutaraldehyde in phosphate buffer (0.1M) for 60 minutes. The samples were treated further with osmium tetroxide (1%) in phosphate buffer (0.1M) for 24 hours. The fixed samples were then dehydrated using 15 minutes transfers through a graded ethanol series followed by propylene oxide.
After the samples were embedded and sliced, the resulting sections were transferred to carbon-reinforced grids. The sections are examined on a Philips CM 12 transmission electron microscope at the Campus Microscopy and Imaging Facility (CMIF) of The Ohio State University.
4.4. Results and Discussion

As a highly conjugated polyene, lycopene in solution is particularly susceptible to isomerization reactions. Whether lycopene cis isomers are formed in vivo, however, is not currently known. Food processing has been postulated to cause isomerization reactions of carotenoids such as β-carotene as a result of thermal treatments of foods (Sweeney and Marsh, 1971; Chandler and Schwartz, 1988; Lessin et al., 1997). However, an increase in lycopene cis isomer percentages was not observed for processed tomato products, (Nguyen and Schwartz, 1998) eliminating thermal processing as a potential causative factor for the presence of lycopene cis isomers in vivo. Table 4.1 reports additional information on the distribution of all-trans lycopene relative to its cis counterparts in tomato products which were produced at the Ohio State University Food Industry Center after an 18-month storage period. It is clear from these results that food processing as well as extended storage did not change the isomer distribution of lycopene. Additionally, the levels of cis isomers in these tomato products remain far below the high levels of cis isomers in humans reported by Clinton et al. (1996).

In contrast to lycopene, a significant amount of β-carotene cis isomers, were formed as tomato products underwent thermal treatment in the retort (Nguyen and Schwartz, 1998) as depicted in Figure 4.1. As a result, we employed five phenotypically different tomato varieties in order to further study the thermal stability of lycopene and β-carotene. The thermal stability of structural intermediates such as δ-carotene and γ-carotene were also examined.
### A. Fresh Tomatoes

<table>
<thead>
<tr>
<th>Types</th>
<th>Description</th>
<th>Lycopene Content (mg/100 g (wet weight))</th>
<th>Percent all-trans isomer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Green</td>
<td>Crimson-type</td>
<td>0.52 ± 0.03</td>
<td>95.8 ± 2.93</td>
</tr>
<tr>
<td>Breaker</td>
<td>Crimson-type</td>
<td>3.84 ± 0.09</td>
<td>94.4 ± 1.49</td>
</tr>
<tr>
<td>Ripe</td>
<td>Crimson-type</td>
<td>5.09 ± 0.08</td>
<td>95.3 ± 2.46</td>
</tr>
</tbody>
</table>

### B. Processed tomatoes and tomato products

<table>
<thead>
<tr>
<th>Types</th>
<th>Description</th>
<th>Lycopene Content (mg/100 g (wet weight))</th>
<th>Percent all-trans isomer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Juice</td>
<td>Heat-concentrated</td>
<td>2.34 ± 0.17</td>
<td>94.0 ± 2.53</td>
</tr>
<tr>
<td>Paste</td>
<td>Heat-concentrated</td>
<td>9.93 ± 0.16</td>
<td>94.9 ± 2.26</td>
</tr>
<tr>
<td>Soup</td>
<td>Retorted</td>
<td>10.72 ± 0.34</td>
<td>95.7 ± 2.15</td>
</tr>
<tr>
<td>Sauce</td>
<td>Retorted</td>
<td>10.22 ± 0.47</td>
<td>94.9 ± 2.19</td>
</tr>
</tbody>
</table>

### C. Processed tomatoes and tomato products after 18-month storage period

<table>
<thead>
<tr>
<th>Types</th>
<th>Description</th>
<th>Lycopene Content (mg/100 g (wet weight))</th>
<th>Percent all-trans isomer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Juice</td>
<td>Heat-concentrated</td>
<td>2.26 ± 0.14</td>
<td>95.8 ± 2.37</td>
</tr>
<tr>
<td>Paste</td>
<td>Heat-concentrated</td>
<td>9.74 ± 0.17</td>
<td>94.7 ± 2.66</td>
</tr>
<tr>
<td>Soup</td>
<td>Retorted</td>
<td>10.50 ± 0.52</td>
<td>95.7 ± 2.98</td>
</tr>
<tr>
<td>Sauce</td>
<td>Retorted</td>
<td>10.29 ± 0.54</td>
<td>94.2 ± 3.23</td>
</tr>
</tbody>
</table>

### C. Biological samples (from Clinton et al., 1996)

<table>
<thead>
<tr>
<th>Types</th>
<th>Description</th>
<th>Lycopene Content (mg/100 g (wet weight))</th>
<th>Percent all-trans isomer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serum</td>
<td>Human</td>
<td>0.032 – 0.102</td>
<td>27-42</td>
</tr>
<tr>
<td>Prostate</td>
<td>Human</td>
<td>0.034</td>
<td>58-73</td>
</tr>
</tbody>
</table>

Table 4.1. Lycopene content and isomer distribution in tomatoes, related-products and biological samples.
Figure 4.1. Impact of thermal processing on lycopene and β-carotene cis isomer percentages.
Figure 4.2 illustrates a typical tomato carotenoid separation using a step-gradient C30 HPLC method. The structure for the eluting carotenoids are also shown. This method enables the separation of lutein, phytoene, phytofluene, β-carotene, δ-carotene, γ-carotene, lycopene and their predominant cis isomers. For the carotenoid prolycopene, a modified protocol is used as it tends to co-elute with β-carotene and other poly-cis lycopene in the above method. Analyses of the tomato varieties illustrate their unique carotenoid profiles (Figure 4.3). In the Crimson variety (RED), which has higher lycopene content then typical commercially-available fruits, the relative abundance of lycopene to other tomato carotenoid is typical (Figure 4.3A). The BET and DEL varieties, likewise, have elevated levels of β-carotene and δ-carotene respectively, making each of these carotenoids the predominant pigment in those varieties (Figures 4.3B and 4.3C). LOW yellow tomatoes yield a reduced total carotenoid profile with β-carotene being the predominant carotenoid (Figure 4.3D). Prolycopene is present in significant levels in PRO tomatoes, even though it accounts for only about a third of the total carotenoids present. Phytoene, phytofluene, and lutein were also monitored for all samples tested. The presence and relative abundance of these minor tomato carotenoids are not of interest in this study other than in terms of chromatographic separation, identification and co-elution.

Upon thermal treatment at 100°C for 30 minutes, increases by an average of 21% and 27% in the cis isomer percentages were observed for β-carotene in RED, PRO, BET, DEL, LOW and lutein in RED, BET, LOW, respectively (Figure 4.4). Lycopene in RED, BET, DEL, prolycopene in PRO, δ-carotene in DEL and γ-carotene in RED,
Figure 4.2. $C_{30}$ separation of major tomato carotenoids.
Figure 4.3. Distribution of selected carotenoids in tomato mutant varieties.

A. Crimpson-type (RED)  B. High-beta (BET)  C. High-delta (DEL)
D. Yellow (LOW)  E. Tangerine-type (PRO)  F. Carotenoid content*.

*The values of 4.3F reflect the sum of only six carotenoids being studied: lycopene, β-carotene, δ-carotene, γ-carotene, lutein and prolycopene. These levels are reported in milligram per 100 gram of tomato tissue (wet weight).
BET, in contrast, remained stable to isomerization in the corresponding mutant varieties. In all the samples where lycopene was present, no significant amounts of lycopene cis isomers were detected in either fresh or heat-treated tissue. δ-Carotene also appeared to remain stable in its all-trans geometrical configuration in the DEL genetic variant. For tomato sections in 80:20 water/olive mixtures which received similar heat treatments, their isomer distributions for all the carotenoids resemble those from the previous heated trial. This observation not only reinforces earlier findings that the presence of oil does not influence the isomerization of all-trans lycopene (Nguyen and Schwartz, 1998) but also extends the trend to all other major tomato carotenoids. Furthermore, the results from this study reveal tomato carotenoids such as lycopene, prolycopene, δ-carotene and γ-carotene have different thermal stability against isomerization reactions relative to β-carotene and lutein.

There are a number of reasons as to why increases in the relative percentage of β-carotene and lutein were observed while lycopene, prolycopene, δ-carotene and γ-carotene, however, remain stable in the all-trans geometrical configuration in all the varieties tested. The first is structural specificity. Differences in molecular shape might determine hydrophobicity, crystalline state and ease of crystal formation, and solubility etc., which in turn can influence thermal stability. For example, lycopene, as a linear molecule, has been shown to likely form multilayers or aggregates (Ray and Misra, 1997) in a Langmuir-Blodgett (LB) film system. Furthermore, once in the aggregated form, lycopene molecules might be able to resist further structural changes. Ray and Misra (1997) reported that upon lowering the surface pressure, which had facilitated the
The reported values are the averages of three trials. Analyses were performed in duplicate on samples from three chromatographic peaks. A: Fresh tomatoes; B: Thermostably-treated samples at 100°C for 300 min.; C: Water. Analysis was performed in duplicate on samples from three chromatographic peaks. A: Fresh tomatoes; B: Thermostably-treated samples at 100°C for 300 min.; C: Water.
formation of lycopene crystal aggregates in the LB film, these microcrystals did not
disintegrate into individual molecules. β-carotene, with two bulky terminal β-ionone
rings, may not be able to easily assemble into an ordered structure (Figure 4.5) and form
as stable a structure as lycopene molecules can.

Furthermore, lycopene, δ-carotene and γ-carotene all have longer chromophores
than β-carotene and lutein with both β-ionone rings. The overall length of the
carotenoid molecule may influence its isomerization stability in terms of activation
energies. Cis isomers of β-carotene and lutein may be thermodynamically stable such
that the tendency to reconvert to the all-trans form is relatively low. Without
experimental data on the relative activation energies, however, any suggestions at this
point are speculations at best. Nonetheless, differences in activation energy for
reversion have been observed for the various β-carotene cis isomers. The activation
energy for trans-cis isomerization about the central carbon-carbon double bond is
reported to be less than those about other double bonds (Zechmeister, 1944). With
respect to prolycopene, while its chromophore is shortened by virtue of possessing four
cis carbon-carbon double bonds, the fact that two of these are hindered cis bonds
(Bartley et al., 1999) renders the possibility of stereochemical-interconversion very
unlikely.

The second and more probable reason behind the different susceptibilities of
tomato carotenoids to thermally-induced isomerization is that these carotenoids are
synthesized and/or stored at different locations in the cell. There is strong evidence
from the literature that the various steps along the carotenoid biosynthetic pathway may
Figure 4.5. Structural features of A. all-trans lycopene and B. all-trans β-carotene.

A.
1. Space-filling model
2. Ball and stick illustration of 1
3. Rotated (90°) view of 2

B.
1. Space-filling model
2. Ball and stick illustration of 1
3. Rotated (90°) view of 2
proceed sequentially at different sites. As a result, tomato carotenoids are associated with different cellular organelles as a function of their biosynthetic order.

During ripening of tomato tissue and chromoplast maturation, active biosynthesis of carotenoids begins and the development of a number of carotenoid-bearing structures commences (Ljubesic et al., 1991). The rapid accumulation of lycopene and increase in concentration results in crystallization (Bathgate et al., 1985). The unique feature of these crystals is that they develop from within the lumina of thylakoids. The crystals remain enveloped by a membrane even after crystallization during the transformation from chloroplast to chromoplast. The uniqueness of this structural characteristic may help to explain the difference in bioavailability of lycopene from fresh tomatoes relative to processed tomato products. The thermal treatments and other unit operations during food processing might be rigorous enough to disrupt cellular walls and organelles. However, it appears that lycopene crystals remain enveloped.

Observations by Rosso (1968) of β-carotene crystals in high-β variety, indicated that the membranes from which these crystals are associated with, appear to be different than those of the lycopene pigment membranes. Cross-sectional view of lycopene crystalloids indicates that they are initiated as plate-like pigment sacs that are formed in association with the thylakoid membrane. The crystalloids increase in size by "involution in preferred planes" to produce tubular crystals resembling the myelin sheaths of nerve cells (Mohr, 1979). In this study, transmission electronmicroscopic examination of tomato samples indicated that in high lycopene and high δ-carotene varieties, crystalloid structures were observed to be associated with the membrane. As
for the high β-carotene varieties, the greater occurrence of plastoglobulin-type
structures is apparent. This is in agreement with previous findings (Ben-Shaul and
Naftali, 1969; Mohr, 1979), which indicated that β-carotene molecules are dissolved in
lipid material of the globules and lycopene is associated with the thylakoid membrane
as crystal deposits.

Figure 4.6 is an electron micrograph of a tomato chloroplast undergoing
transformation to a chromoplast in fresh Crimson-type tissues. At 50,000X
magnification, the globular structure, to which β-carotene is associated, and the
thylakoids, making up the granal stack, are visually well defined. Figure 4.7, on the
other hand, is an electron micrograph of lycopene crystalloids in a mature chromoplast
of fruit tissue from the same tomato variety. While an attempt was not made in this
study to isolate the various tomato organelles and analyze their carotenoid content, it
was possible to directly correlate the electronmicrographic information with HPLC
determinations of extracted carotenoids as well as light microscope observations.

The use of mutant varieties having only one carotenoid as the major pigment
allowed for the tentative characterization and classification of colored organelles. In
red tomatoes, for example, thylakoid-associated crystalloids are considered lycopenic as
a result of their red color when observed under a light microscope. Additionally, Ben-
Shaul and Naftali (1969) have shown that the majority of the crystalloids isolated from
red tomatoes are lycopene crystals by electron-diffraction. In the LOW variety where
lycopene is not present, these red thylakoid-associated crystalloids were not observed.
In fact, only globular pigment bodies were found.
Figure 4.6. Electron micrograph of chloroplast from green tomatoes tissue undergoing ripening. A. Plastoglobulin-type structure. B. Thylakoids.
Figure 4.7. Electron micrograph of lycopene crystalloids in mature red tomato chromoplast. A. Lycopene crystalloids. B. Plastoglobulin-type sacs in which β-carotene reportedly accumulates.
Similarly, in BET and LOW, the density of globular bodies believed to contain β-carotene crystals dissolved in lipid material were observed to be a function of the tissue’s β-carotene content as determined by HPLC. Ultrastructural examination of the Jubilee (tangerine-type) tomatoes’ chromoplasts, on the other hand, was inconclusive, as prolycopene is not the only predominant carotenoid in these tomatoes (Bartley et al., 1999). Nonetheless, Rosso (1967) has noted that Golden Jubilee chromoplasts are classified as those containing crystallized carotenoids. At this time, we are suggesting that, since prolycopene in PRO, like lycopene in RED, did not isomerize and that these two carotenoids have similar biosynthetic order, prolycopene probably is also protected from conformational changes in the same fashion as lycopene. Furthermore, the presence of hindered cis bonds also make the geometrical conversion to the trans configuration sterically prohibitive.

Ultimately, tomato carotenoids have been shown to have significantly different susceptibility against thermal isomerization. Upon thermal treatment, β-carotene and lutein isomerize to a greater extent than δ-carotene, γ-carotene and lycopene. The presence of lipid did not influence the extent or likelihood of lycopene and other tomato carotenoids in the all-trans configuration to isomerize. The overall carotenoid content did not change significantly, as fresh tomato samples were heat treated under typical food preparation and thermal processing conditions. Differences in tomato product formulation, severity of heat treatments, the presence and varying levels of other carotenoids did not influence the formation of lycopene cis isomers. Electron micrographs of heat-treated samples compared to those of fresh tomato tissues revealed that thermal processing imparts changes to the physical structure of tomato tissues.
Despite these changes, which include cell wall and organelle deformation as a result of heat and shear during processing, lycopene's isomerization stability was not affected.

Thus, while thermal processing reportedly enhances the bioavailability of lycopene (Sies and Stahl, 1992, Gardner et al., 1997), it does not alter the chemical nature of the molecule itself. The results not only illustrate the difference in stability of all-trans lycopene against thermal isomerization relative to β-carotene but also suggest structural and cellular factors which might be responsible for the observed difference. Ultimately, this study eliminates several factors associated with food processing and storage as likely causative agents of the elevated levels of lycopene isomers associated with biological samples. Further studies are needed to determine whether the differences in susceptibility to isomerization reactions among tomato carotenoids are due to other processes between ingestion and tissue deposition phases of lycopene metabolism.
4.5 References


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4.6. Acknowledgements

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The authors also thank Dr. Lane Sander of the National Institute of Standards and Technology, Gaithersburg, MD for providing the polymeric C₃₀ columns used in this project.
Chapter 5

ISOMERIZATION OF ALL-TRANS LYCOPENE AND OTHER TOMATO CAROTENOIDS FOLLOWING IN VITRO DIGESTION


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5.1. Abstract

Factors governing the higher ratio of cis to trans lycopene isomers in human biological fluids and tissues relative to those in the diet remain unclear. Using an in vitro digestive system, we monitored the extent of lycopene isomerization prior to and following absorption by Caco-2 human colon cells for several different lycopene sources including tomatoes, rosehip puree, lycopene supplement pills, beadlet, microfiber and lycopene standards in applesauce. Fresh tomatoes of five different carotenoid compositions were also subjected to digestion: normal red, reduced total carotenoid, high β-carotene, high δ-carotene and tangerine-type containing a high level of prolycopene. All samples were normalized to contain 10% vegetable oil before digestion.

C30 reversed phase HPLC analysis of micellarized carotenoids from digesta fractions revealed that lycopene is stable in both the gastric and intestinal phases of digestion. Likewise, the geometrical isomer distribution of other tomato carotenoids including prolycopene, β-carotene, δ-carotene and γ-carotene also remain the same. These findings suggest that the ratio of cis-trans isomers for predominant tomato carotenoids including lycopene remains the same beyond ingestion at least until the end of the digestive process. The results also indicate that the likelihood of cis- isomers of lycopene and other carotenoids being incorporated into micelles is similar to their all-trans counterparts. The isomer distribution of lycopene and other tomato carotenoids began to shift towards a higher level of cis isomers upon uptake by Caco-2 cells suggesting a cellular mechanism to the accumulation of carotenoids in the cis configuration. This hypothesis is further substantiated by observations of a higher
percentage of lycopene cis isomers (~60%) in human blood chylomicron fractions from individuals consuming tomato sauce with a cis isomer level of ~5%.

Thus, the high level of lycopene cis-isomers observed in human serum and select tissues, is more likely the product of processes associated with cellular uptake. The mechanism responsible for the observed increases of lycopene cis-isomer percentages in Caco-2 cells remains to be elucidated. The possibilities include: preferential uptake of cis-isomers across the cell membrane, isomerization of the all-trans isomer following absorption and cellular processes that are shape-selective or solubility-dependent.
5.2. Introduction

Accumulating epidemiological evidence has revealed inverse associations between tomato intake or blood lycopene level and the risk of several cancers (Block et al., 1992; van Poppel, 1993; Steinmetz and Potter, 1996; Giovannucci, 1999). These findings, in turn, have prompted considerable interest in lycopene and its stability in food. Furthermore, the fact that the distribution of lycopene geometrical isomers in the diet is markedly different from that of human biological samples such as serum and tissues (Clinton et al., 1996; Schierle et al., 1997) is also particularly intriguing in the context of physiological implications of lycopene (Nguyen and Schwartz, 1999). Understanding the various factors which affect lycopene's isomer distribution in the diet and in humans, therefore, is an important step towards a better appreciation for the role and implications of this carotenoid in the diet and human health.

It has previously been assumed that the higher percentage of lycopene cis-isomers in human biological samples is due in part to consumption of heat treated tomato products containing cis-isomers of lycopene (Stalh and Sies, 1992, Gartner et al., 1997, Schierle et al., 1997, van het Hof et al., 1998). However, recent findings from our laboratory have indicated that typical food processing and storage of tomato products have no impact on the isomer distribution of various dietary lycopene sources, regardless of product type, container type, moisture content, tomato variety, and severity of heat treatment (Nguyen and Schwartz, 1998). These findings suggest that the high ratio of cis to trans-lycopene isomers in human biological samples must have formed or become concentrated during digestion, absorption or uptake into the bloodstream.
In order to test the hypothesis that lycopene isomerization occurs after ingestion of tomatoes and related products, an *in vitro* digestion model has been applied to a variety of lycopene sources in this study. Tomato carotenoids including lycopene from various sources will be subjected to an *in vitro* digestion process which mimics the gastric and intestinal phases of digestion. Subsequently, the aqueous micellarized carotenoid fractions of the digesta will be introduced to Caco-2 cells in order to assess the geometrical isomer profile of carotenoids traversing a biological membrane. All the samples will be analyzed via a polymeric C$_{30}$ reversed-phase HPLC method optimized for the separation of all major tomato carotenoids and their predominant geometrical isomers.

The *in vitro* digestion model presents a rapid and reliable alternative to the use of human and animal subjects to assess the stability as well as relative bioavailability of food components. This technique has been successfully employed for the *in vitro* assessment of the bioavailability of amino acids (Linberg *et al.*, 1998), cholesterol (Fouad *et al.*, 1991), iron (Gangloff *et al.*, 1996), phosphorus (Liu *et al.*, 1998), vitamin B6 (Ekanayake *et al.*, 1986) as well as of carotenoids (Garret *et al.*, 1997). The digestive phases of the procedure successfully transferred lipophiles such as carotenoids from the food matrix to the micellar phase. The use of the Caco-2 cell line, likewise, provides a biological reference for the absorption of carotenoids by intestinal mucosal cells. Ultimately, the coupling of the digestive protocol with the Caco-2 cell uptake analysis will allow for the monitoring of isomer distribution of carotenoids destined to be transported to the bloodstream.
The amount of lycopene cis isomers in blood chylomicron fractions from individuals consuming tomato products were also measured to collaborate findings from the in vitro experiments. Table 5.1 illustrates how the findings of this study will fill the information gap regarding when lycopene isomerization begins. These findings will determine whether digestion and intestinal absorption are important time points in terms of the accumulation of lycopene cis isomers. The information will be useful in assessing both the nutritional and physiological implications of this emerging phytochemical in the diet as well as in helping to explain its behavior and stability in vivo.
<table>
<thead>
<tr>
<th>Time point</th>
<th>Sample description</th>
<th>Percent lycopene cis isomers</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biosynthesis</td>
<td>Tomato Fruits</td>
<td>4.2-5.6</td>
<td>Zechmeister et al., 1941 Emenhiser et al., 1996 Chapter 4</td>
</tr>
<tr>
<td>Processing</td>
<td>Tomato Products</td>
<td>4.3-6.0</td>
<td>Nguyen and Schwartz, 1998</td>
</tr>
<tr>
<td>Storage</td>
<td>Tomato Products</td>
<td>4.2-5.8</td>
<td>Chapter 4</td>
</tr>
<tr>
<td>Ingestion</td>
<td>Masticated Food</td>
<td>Unknown</td>
<td>None</td>
</tr>
<tr>
<td>Digestion</td>
<td>Gastric Digesta</td>
<td>Unknown</td>
<td>None</td>
</tr>
<tr>
<td></td>
<td>Intestinal Digesta</td>
<td>Unknown</td>
<td>None</td>
</tr>
<tr>
<td>Absorption</td>
<td>Chylomicron</td>
<td>Unclear</td>
<td>Gardner et al., 1997</td>
</tr>
<tr>
<td>Deposition</td>
<td>Prostate Tissue</td>
<td>58-73</td>
<td>Clinton et al., 1996 Stahl et al., 1993</td>
</tr>
</tbody>
</table>

Table 5.1. Distribution of lycopene cis isomers from biosynthesis to absorption and distribution.
5.3. **Materials and Methods:**

Standards of all-trans β-carotene and lycopene were purchased from Sigma Chemical Co. (St. Louis, MO). All extraction and HPLC solvents (Fisher Scientific, Fairlawn, NJ) were certified HPLC or ACS grade. Ammonium acetate (Fluka; Ronkonkoma, NY) was dissolved in water and adjusted to pH 4.6 with glacial acetic acid to make a 1.0 mol L⁻¹ solution. Dulbecco’s minimum essential medium (DMEM), fetal calf serum, L-glutamine, amphotericin B, gentamicin, nonessential amino acids, sodium bicarbonate, N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES), pepsin, pancreatin (porcine), bile extract (porcine) and sodium taurocholate were purchased from Sigma Chemical Co. (St. Louis, MO). L-(4,5-²H) leucine (specific activity 171 Ci/mmol) was purchased from Amersham Life Sciences Inc. (Arlington Heights, IL). Alamar blue dye was obtained from Alamar Biosciences, Inc. (Sacramento, CA). All other reagents and materials were purchased from Fisher Scientific Co. (Norcross, GA).

Five tomato varieties with different color and carotenoid composition were used: high-lycopene (LYC), high β-carotene (BET), high δ-carotene (DEL), tangerine-type tomatoes (PRO) with prolycopene as the predominant carotenoid and yellow tomato with reduced polyenes (LOW). These tomatoes were kind gifts from Dr. David Francis of the Horticulture and Crop Science Agriculture Research and Development Center in Wooster, Ohio. In another trial, lycopene from sources such as Crimson-type (RED), tangerine-type tomatoes (POL), guava concentrate (GUA), lycopene supplement pill (PIL), lycopene beadlet (BEA), lycopene microfiber (FIB), and rosehip puree (ROS) were tested. All samples were adjusted to contain 10% oil and applesauce was used as a bulking agent whenever appropriate to facilitate dispersion and uniformity. Samples
were homogenized and cooled on ice until the commencement of the in vitro digestion experiments.

5.3.1. In vitro Digestion Experiments:

The digestion protocol, consisting of gastric and intestinal phases, was a modification of that described by Miller et al. (1981).

**Gastric phase:** The sample was acidified to pH 2 using 1 M HCl. Two milliliters of porcine pepsin (40 mg/ml suspension in 0.1 M HCl) were then added and the mixture was incubated in amber vials at 37°C for 1 hour in a water bath shaking at 95 rpm. Following incubation, the pH was adjusted to 5.3 using 0.9 M sodium bicarbonate.

**Intestinal phase:** Nine milliliters of bile extract-pancreatin mixture (2 mg/ml of pancreatin and 12 mg/ml bile extract in 100 mmol/L sodium bicarbonate) was added to the gastric digesta. Final concentrations of pancreatin and bile extract in the reaction mixture were 0.4 mg/mL and 2.4 mg/mL, respectively. Ten milliliters of this mixture were transferred to 10 ml amber vials. The pH was adjusted to 7.5 using 1 N NaOH and the digesta was overlaid with argon. The deaerated and sealed sample was incubated at 37°C for 2 hours in a waterbath shaking at 95 rpm. The digesta, at the completion of the intestinal phase of the in vitro digestion process, consisted of three phases: the aqueous fraction containing micelles, a lipid phase comprising of residual oil droplet and a solid phase made up of undigested food particles.

**Micellar fractionation:** The digesta was fractionated according to Hernell et al. (1990) into an aqueous portion containing micelles and non-digested materials using a 50.3 Ti rotor and the Beckman L7-65 Ultracentrifuge at 167,000 x g at 4°C for 95 minutes. The
aqueous micellar fraction was passed through a 0.22μm filter, collected and stored at -80°C for subsequent extraction and storage.

**Caco-2 cell culture:** Caco-2 cells were obtained from American Type Culture Collection (Rockville, MD) at passage number 19 and stock cultures were maintained as previously described (Hildago et al., 1989; During et al., 1998). Complete medium contained high glucose DMEM (Sigma Chemical Co.), 10% heat-inactivated fetal calf serum (FCS), nonessential amino acids (10 mL/L medium), L-glutamine (2mmol/L), amphotericin B (0.5 mg/L), gentamicin (50 mg/L), HEPES (15 mmol/L), and sodium bicarbonate (44 mmol/L). Cells were grown in 12-well plastic dishes (Beckton Dickinson Labware, Franklin Lakes, NJ) in a humidified atmosphere of air/CO₂ (95:5, v:v) at 37°C. Fresh complete medium was added every 2 days. Cultures were used for experiments between 11 and 14 days post-confluency when Caco-2 cells have been observed to exhibit maximum differentiation as assessed by the activities of the marker enzymes alkaline phosphatase and sucrase (Ellwood et al., 1993).

**Cellular uptake of micellar carotenoids:** Uptake experiments were performed using Caco-2 cultures between the 23rd and 37th passage. Monolayers were washed twice with 1 mL Hank's balanced salts solution (HBSS) before adding 1 mL of test medium containing the aqueous fraction from the *in vitro* digestion procedure that had been diluted 1:3 (v:v) with basal DMEM. Cultures were incubated at 37°C and harvested 4 hours later. After removing spent medium, monolayers were washed three times with HBSS containing 5 mmol/L sodium taurocholate at 22°C and collected in 1 mL ice-cold phosphate buffered saline containing 10% (v:v) ethanol and 45 mmol/L BHT. Samples were then centrifuged
into cell-pellets and stored at -80°C under a blanket of argon until analysis. Experiments are done in triplicate and analysis of each sample were performed in duplicate.

5.3.2. Chylomicron Isolation.

Blood from two healthy, non-smoking, adult females was drawn at time 0 and 3 hours after consuming 4 medium size tomatoes with buttered bread for breakfast. These individuals have been on a lycopene-free diet for three days before the start of the experiment. Blood samples were immediately centrifuged at 4°C for 10 minutes at 3000 rpm and serum was transferred in 1.0 mL aliquots to microcentrifuge tubes. Chylomicron fractions were obtained via a modified method described by Terpstra, 1985 by layering 3.7 mL of saline solution (d= 1.0063) over 0.8 ml of plasma and centrifuging the layers at 33,500 rpm in a SW50.1 swinging bucket rotor for 30 minutes at 4°C. The top creamy film was removed and stored at -80°C until HPLC analysis.

5.3.3. Extraction and Saponification.

**Fruit and vegetable samples:** The following extraction procedure was carried out under subdued light to prevent isomerization and photodegradation as previously described (Schwartz and Nguyen, 1997). Fresh and processed tomatoes were diced and 10.0 g samples were homogenized in 50 mL methanol with 1.0 g CaCO₃ and 3.0 g Celite. Samples were successively extracted with mixture of 1:1 acetone/hexane and vacuum-filtered through Whatman paper No. 1 and 42. The filtrates were combined in a separatory funnel and water was added to induce phase separation. The hexane layer was removed and brought up to volume. Triplicate 5 mL samples were dried by flushing with nitrogen.
Extracts were analyzed in duplicate by reversed-phase HPLC. Extracts of samples containing lipids was saponified with 30% KOH for 60 minutes before aliquots of the filtrate were dried.

**Serum, chylomycron and micellarized fraction extraction:** Carotenoids were extracted successively with three 2.0-mL aliquots of acetone: hexane (50:50) containing 0.02% (w/v) (BHT). The solution was vortexed for 30 sec. The sample was then centrifuged at 10,000 x g for 1 min after which the hexane layer was decanted off and saved. The combined extracts were evaporated under nitrogen and analyzed immediately.

**Caco-2 cell extraction:** Cell extraction was performed by the method of Peng and Peng (1992). Carotenoids were extracted by addition of 200 μL of protease solution (100mg protease/10mL PBS) to the thawed cells. The sample was vortexed and incubated at 37°C for 30 minutes. Following digestion, 400μL of SDS/ethanol solution (1.0 mL of 20% SDS/water solution into 19.0 mL ethanol) was added and vortex-mixed. Subsequently, 500μL of hexane : acetone (3:1) containing 0.2% BHT was added and vortexed. The sample was then centrifuged at 10,000 x g for 1 min after which the hexane layer was decanted off and saved. The hexane:acetone extraction was repeated once. The combined hexane layers were dried under a stream of nitrogen, redissolved in mobile phase and injected.

**Prostate tissue extraction:** Approximately 10 mg of prostate tissue samples were taken from storage (-80°C in saline) and thawed. Each sample was rinsed with 1.0 mL of fresh saline. The tissue was weighed and placed in an 8-mL vial containing 1.8 mL of saline with 1000 units of collagenase and 0.2 mL of saline with 2.5 g/L of ascorbic acid.
The sample was ground by mechanical homogenization and incubated at 37 °C for 60 min, after which 2.0 mL of 5.0% (w/v) ethanolic KOH was added. The sample was then saponified for 60 min at 37 °C. Carotenoids were extracted successively with three 2.0 mL aliquots of hexane containing 0.02% (w/v) (BHT). The solution was vortexed for 30 seconds and the hexane layer collected and saved. The combined hexane layers were evaporated under nitrogen and stored dry until prepared for analysis as described below.

**Recovery determinations:** Results of recovery determinations during extraction, saponification and chromatography procedures were found to be greater than 95%. *Cis*-isomer content of samples with added all-trans carotenoid standards remained the same as control extracts, indicating that artifactual isomerization did not occur during the handling of these samples and subsequent chromatographic procedures.

### 5.3.4. HPLC Analysis

**Instrumentation and chromatography for tomato tissues, digesta and micellar fractions:** The reversed-phase HPLC system used in this study consisted of a Waters (Milford, MA) 2690 separation module. Separations were achieved using analytical (250 x 4.6 mm I. D.) 3μm polymeric C30 columns which were prepared at the National Institute of Standards and Technology (Gaithersburg, MD) according to Sander et al., 1994. Guard columns packed with C30 stationary phase were used in-line for all separations. Lycopene isomer separation was carried out as reported earlier (Schwartz and Nguyen, 1997) at 1.0 mL per minute using a linear gradient of 40 to 50% methyl-\(r\)-butyl ether (MTBE) in methanol for 35 min. Total carotenoid separation was carried out
at 1.0 mL per minute using a multi-step linear gradient of 15 to 50% MTBE in methanol for 55 min.

**Instrumentation and chromatography for Caco-2 cell and other biological samples:** An ESA model 5600 Coularray electrochemical detector (Chelmsford, MA) equipped with either four or eight channels in series was used for analyses. The potential settings from channel 1 - 8 were 100 mV to 520 mV in 60-mV increments. For lycopene analysis only four channels were used, set at 220mV to 520 mV in 100 mV increments. Separations for all carotenoids except lycopene were achieved using gradient elution with different concentrations of methanol-MTBE-ammonium acetate in reservoirs A (95:3:2) and B (25:73:2). The following gradient was used: 0 to 5 min, 87.5% A, 12.5% B; 5 to 25 min, linear gradient to 65% Solvent A, 35% solvent B; hold 5 min. Lycopene and lycopene isomer separation was achieved isocratically in 50.0 min with a solvent system composed of 45% A and 55% B (Ferruzzi et al., 1998).

**5.3.4. Peak Identification:**

**Iodine isomerization:** β-Carotene and lycopene standards along with purified δ-carotene, γ-carotene, and lutein extracts were solubilized in 2 mL of hexane and treated with 10 μL of a 1.0% iodine in hexane solution. Following brief exposure to direct sunlight, the mixture was dried, resuspended in mobile phase and injected. The resulting separations were used to tentatively identified cis isomers of the corresponding carotenoids being evaluated.

Column effluent was monitored via a Waters 996 Photodiode Array Detector at 200-800 nm with a scanning rate of 2 scan/second and 1.2 nm spectral resolution. The
detector was linked to a Digital (Maynard, MA) S133 Venturis computer with Waters Millennium 2010 chromatography software (LC Version 2.15.01). Quantification of geometric isomers of carotenoids was achieved using a standard curve of the corresponding all-trans isomers and their molar absorptivity coefficients (Britton, 1995). This method approximates the cis-isomer content, as the molar extinction coefficient values for individual isomers are not known. In the case of the poly-cis lycopene isomer in Tangerine tomatoes, the molar absorptivity is known. Chromatographic peak identification was based on comparison to previously reported separations on polymeric C_{30} columns and UV-visible absorption spectral libraries. Peaks which were identified as cis-lycopene isomers had been ascertained using electrospray mass spectroscopy (van Breemen, 1996; Emenhiser et al., 1996; Clinton et al., 1996) to have the same molecular weight as all-trans lycopene and thus are not oxygen-addition products. The identities of all-trans isomer peaks were assigned based on retention time and co-chromatography of authentic standards as well as by comparing the observed maximum absorptivity in two separate solvents system against published values. Tentative identifications are made for the remaining carotenoids cis isomers by comparing their retention time and spectrum to the iodine photomutation mixture as well as the appearance of the “cis-peaks” in the ultraviolet region of the spectra.
5.3. Results and Discussion

The mechanism for the shift towards higher percentages of lycopene cis-isomers in human serum and tissues compared to those of fresh fruits and vegetables is currently not known and remains open to speculation. In order to determine whether the accumulation of lycopene isomers begins before or after entering into the circulation, three types of biological samples, chylomicron fraction, serum, and prostate tissue were analyzed. The chylomicron fraction, isolated from blood samples collected 3 hours following ingestion of 4 medium-size tomatoes with buttered bread for breakfast, contains postprandial lycopene. Lycopene from serum and prostate samples, on the other hand, represents circulating and deposited levels.

Analysis of all these biological samples revealed significantly higher percentages of lycopene cis isomers in these samples than that of dietary sources such as fresh and processed tomato sauce (Figure 5.1). The distribution data for serum and prostate analyses, 59.4% and 77.8% cis lycopene isomers respectively, is in agreement with earlier findings by Clinton et al. (1996). Moreover, this information suggests that lycopene in serum and tissue may have been formed prior to its entry into the circulation. Assessment of lycopene cis-trans isomer distribution in the chylomicron fraction, substantiates this hypothesis. With 66.2% of the postprandial lycopene in the cis configuration, this higher levels of cis isomer compared to that of tomato products indicates that isomerization of lycopene may be a function of digestive or absorptive processes prior to its transfer to the lymphatic system and blood circulation. Earlier findings by Gartner et al. (1997) also reported high percentages of lycopene cis isomers, agreeing with these results.
Figure 5.1. C₃₀ separation of all-trans lycopene and its geometrical isomers.

A. 1. Fresh tomato, 2. Tomato sauce  
B. Chylomicron fraction  
C. Serum  
D. Prostate tissue.  
** E = All-trans lycopene, 5-Z = lycopene 5-cis isomer.
In the next phase of the study, the *in vitro* digestion technique was used to assess lycopene isomerization during digestion. We employed five phenotypically-distinct tomato varieties containing different levels of lycopene and other structurally-related carotenoids such as prolycopene, β-carotene, δ-carotene and γ-carotene (Figure 5.2). The high levels of key individual carotenoids in each mutant variety facilitated the unique opportunity to evaluate their relative abundance in fresh tissue as well as making it easier to quantify chromatographically any *cis* isomer formed following digestion. The marked differences in carotenoid content also provided useful comparative points of reference in terms of possible tissue matrix effect, concentration dependence and carotenoid interactions.

HPLC analytical results illustrate the unique carotenoid profiles of these tomato varieties (Figure 5.3). In the LYC variety, which has higher lycopene content than typical RED (Crimson-type) fruits, the relative abundance of lycopene to other tomato carotenoids is not unusual (Figure 5.3A). The BET and DEL varieties, likewise, have elevated levels of β-carotene and δ-carotene respectively, making each of these carotenoids the predominant pigment in those varieties (Figures 5.3B and 5.3C). LOW yellow tomatoes yield a reduced total carotenoid profile with β-carotene being the predominant carotenoid (Figure 5.3D). Even though prolycopene is not the most predominant carotenoid in the PRO variety, its unique structure containing two hindered *cis* double bonds makes it useful as a marker. The approximate total carotenoid content is given for each mutant variety in Figure 5.3F.

In RED samples, lycopene was found to remain stable throughout the *in vitro*
- **Lycopene** ($\psi, \psi$-carotene, $C_{40}H_{56}$)

- **Prolycopene** (7,9,9',7'Z-lycopene, $C_{40}H_{56}$)

- **$\gamma$-carotene** ($\beta, \psi$-carotene, $C_{40}H_{56}$)

- **$\delta$-carotene** ($\epsilon, \psi$-carotene, $C_{40}H_{56}$)

- **$\beta$-carotene** ($\beta, \beta$-carotene, $C_{40}H_{56}$)

Figure 5.2. Structure of predominant tomato carotenoids monitored for isomerization in this study.
A. LYC

B. BET

C. DEL

D. LOW

E. PRO

F. CAROTENOID CONTENT

Figure 5.3. Carotenoid content in the five tomato mutant varieties used in this study. The values reflect the sum of the six carotenoids being studied: lycopene, β-carotene, δ-carotene, γ-carotene, lutein and prolycopene. These levels are reported in milligram per 100 gram of tomato tissue (wet weight).
Figure 5.4. Lycopene’s isomer distribution as determined by C\textsubscript{30} chromatography of LYC tomato sample throughout the \textit{in vitro} digestive process and Caco-2 cellular uptake.
digestive process (Figure 5.4). During both the gastric and intestinal phases of digestion, the isomer distribution remains relatively the same (93:7 and 91:9 respectively) as that of the starting material (95 all-\textit{trans}: 5 total \textit{cis}). The incorporation of lycopene into micelles did not change the \textit{trans-cis} distribution at 93:7. This trend is applicable to \(\beta\)-carotene and \(\gamma\)-carotene in the RED sample as well.

When tomato samples from the five mutant varieties (LYC, PRO, BET, DEL and LOW) were subjected to the \textit{in vitro} digestive process, increases in the \textit{cis} isomer percentages were not observed for \(\beta\)-carotene (LYC, PRO, BET, DEL, LOW), prolycopene (PRO), \(\delta\)-carotene (DEL) and \(\gamma\)-carotene (LYC and BET) during gastric and intestinal digestive phases. In all the samples where lycopene was present, no significant amounts of lycopene \textit{cis} isomers were detected in either gastric or intestinal phases of the digestive process (Table 5.2). These results reinforce findings in RED that the isomerization of all-\textit{trans} lycopene is not affected by digestive processes. Furthermore, the results from this part of the study reveal that other tomato carotenoids such as prolycopene, \(\beta\)-carotene, \(\delta\)-carotene and \(\gamma\)-carotene have similar stability to lycopene towards isomerization reactions under \textit{in vitro} digestive conditions. The extremes in pH values and their fluctuation during the digestive process had been suggested to affect the stability of carotenoids (Khachik \textit{et al.}, 1992b). According to this \textit{in vitro} study, in which the pH varies from 2 to 7.5, tomato carotenoids were found quite stable throughout the entire digestive process.

Overall, lycopene, prolycopene, \(\beta\)-carotene, \(\delta\)-carotene and \(\gamma\)-carotene all remained stable in the all-\textit{trans} geometrical configuration during the digestive process for
all the tomato samples evaluated in this study. Lycopene isomer distribution, however,
shifted significantly from 93:17 in the isolated micellar fraction to 59:41 (net change of
34%) within the Caco-2 cell samples. Much lower amounts of cis isomers were detected
for the other carotenoids tested in the remaining Caco-2 cells samples. These net changes
range from 5.4 for γ-carotene, 4.9 for β-carotene and 4.8 for δ-carotene. The relative
abundance of prolycopene in the starting material and in the cells remained constant
(Table 5.2). This finding can be explained in the context of the resistance to
isomerization of prolycopene’s hindered cis bonds (Englert, 1979). Electron micrographs
of digested samples compared to those of fresh tomato tissues revealed that the in vitro
digestive process imparts changes to the physical structure of tomato tissues as
anticipated. The tomato tissue matrix is significantly altered by digestion but apparently
not enough to have lost its ability to protect the carotenoids from the digestive
environment.

In the last phase of this study, lycopene from a variety of sources were subjected
to the same in vitro digestion model and Caco-2 cell uptake protocol (Figure 5.5).
Lycopene in these samples provides an excellent assessment of its stability in different
physical states, which might be found in the diet. Tomato sauce was used to compare
with fresh tomatoes. Lycopene beadlets, microfiber, and supplement pill were also used
to examine these various forms of lycopene that may be used in human and animal
lycopene bioavailability studies. Finally, guava and rosehip samples were used to
provide additional comparative benchmarks against fresh tomatoes. The guava sample
was concentrated to elevate the overall lycopene concentration. It is important to note
that the concentration process of guava by heating at 100°C for 90 minutes did not induce
<table>
<thead>
<tr>
<th>Carotenoid</th>
<th>Mutant Variety</th>
<th>Raw</th>
<th>Micelle</th>
<th>Caco-2</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>All-trans</strong> Lycopene</td>
<td>High-Lycopene (LYC)</td>
<td>90.9</td>
<td>90.3</td>
<td>61.4</td>
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<tr>
<td>Prolycopene</td>
<td>Yellow (POL)</td>
<td>36.5</td>
<td>43.8</td>
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<td>High-Delta (DEL)</td>
<td>87.7</td>
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<td>83.8</td>
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<td>85.7</td>
<td>80.8</td>
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<tr>
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<td>High-Beta (BET)</td>
<td>91.2</td>
<td>89.3</td>
<td>83.9</td>
</tr>
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Table 5.2. Percentages of all-trans carotenoids in tomato tissues following *in vitro* digestion and Caco-2 cell uptake.
Figure 5.5. Lycopene isomer distribution as determined by C₃₀ chromatography
A. Starting materials, B. *In vitro* digested samples and C. Caco-2 cells.
any thermal isomerization. This is in agreement with our previous findings (Nguyen and Schwartz, 1998; Nguyen and Schwartz, 1999) of lycopene stability against thermal processing in tomato products. It is, however, in contrast to a report observing lycopene cis isomers in processed guava juice by Padula and Rodriguez-Amaya (1987).

For all the samples tested except ROS, the isomer distribution remained unaffected by the in vitro digestion process. In the PIL sample, which has a high initial percentage of cis-isomers (53.2%), an increase in that percentage was not observed. The only significant change was in the ROS sample. Upon uptake by Caco-2 cells, the level of lycopene isomers rises in all the samples studied, confirming earlier results that during or following absorption by the cells, isomerization reactions appear to have occurred (Figure 5.4).

The overall carotenoid profiles of Caco-2 cells in all samples consisted of a significant amount of the corresponding cis isomers. The interconversion of carotenoid geometrical configuration may have been induced by certain cellular constituents that were capable of releasing the π-bond of carbon atoms involved in double bonds. The free rotation about the axis of the remaining single σ-bond between these carbons, carrying substituted groups, and subsequent reformation of the double bond results in a change in configurational geometry (Zechmeister and Tuxson, 1939). This possibility, however, is strictly speculative, and is based upon observations of lycopene isomerization as a result of exposure to sensitizers (Nguyen and Schwartz, 1998).

Another possibility for the rise in cis isomer percentages in Caco-2 cells is the fact that carotenoids in the cis configuration are more soluble in a lipid environment than their all-trans counterpart (Zechmeister, 1962). Cis isomers, having a bent shape, are also less
likely to form an ordered structure such as crystals or aggregates. These two factors may in turn dictate the availability of carotenoids in the cis configuration for absorption via diffusion and passive transport through the cell membrane. Cis isomers of carotenoids, therefore, might accumulate preferentially over the all-trans forms. This possibility is more probable than the assumption of the presence of isomerases that act upon the all-trans form of these carotenoids. As specific physiologic functionality has not been discovered for any carotenoid cis isomers other than the debatable case of 9-cis β-carotene (Heyman, 1992), the presence of these enzymes is unlikely. In the case of prolycopene in PRO sample, as have been discussed earlier, a change in its relative percentage was not expected and not observed. The hindered cis bonds of prolycopene would have difficulty undergoing cis-trans interconversion due to steric hindrance.

While the specific isomerization mechanism of lycopene and other tomato carotenoids remains to be elucidated, the findings from this study have essentially shortened the list of possible sites where isomerization may have initiated (Table 5.3). The results provide compelling evidence that the intestinal mucosal cells are the most likely anatomic site where the isomer distribution of lycopene, specifically, begins to shift towards higher cis isomer percentages. The elevated level of lycopene cis isomers is observed in subsequent steps of lycopene metabolism - in the chylomicron fraction, in the circulation and even upon deposition and storage in tissue such as the prostate gland. At the present time, we cannot exclude the possibility that some cis-isomers of lycopene and the other carotenoids may have formed during the four hours incubation period with Caco-2 cells. Our conclusion that the intestinal mucosal is the site of isomer distribution shift, nonetheless, is not compromised by this possibility.
<table>
<thead>
<tr>
<th>Types</th>
<th>Description</th>
<th>Total cis isomer percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>1. Biosynthesis</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Green</td>
<td>Crimson-type</td>
<td>4.2</td>
</tr>
<tr>
<td>Breaker</td>
<td>Crimson-type</td>
<td>5.3</td>
</tr>
<tr>
<td>Ripe</td>
<td>Crimson-type</td>
<td>4.7</td>
</tr>
<tr>
<td><strong>2. Processing</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Juice</td>
<td>Heat-concentrated</td>
<td>6.0</td>
</tr>
<tr>
<td>Paste</td>
<td>Heat-concentrated</td>
<td>5.7</td>
</tr>
<tr>
<td>Soup</td>
<td>Retorted</td>
<td>4.3</td>
</tr>
<tr>
<td>Sauce</td>
<td>Retorted</td>
<td>5.1</td>
</tr>
<tr>
<td><strong>3. Storage</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Juice</td>
<td>Heat-concentrated</td>
<td>4.2</td>
</tr>
<tr>
<td>Paste</td>
<td>Heat-concentrated</td>
<td>5.3</td>
</tr>
<tr>
<td>Soup</td>
<td>Retorted</td>
<td>4.3</td>
</tr>
<tr>
<td>Sauce</td>
<td>Retorted</td>
<td>5.8</td>
</tr>
<tr>
<td><strong>5. Digestion</strong></td>
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<td></td>
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<tr>
<td>Digesta</td>
<td>Gastric</td>
<td>7.3</td>
</tr>
<tr>
<td>Digesta</td>
<td>Intestinal</td>
<td>9.4</td>
</tr>
<tr>
<td>Micelle</td>
<td>Fractionated</td>
<td>7.2</td>
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<tr>
<td><strong>6. Absorption</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Caco-2 cells</td>
<td>Culture</td>
<td>62.9</td>
</tr>
<tr>
<td>Chylomicron</td>
<td>Human</td>
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<tr>
<td><strong>7. Deposition</strong></td>
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<td></td>
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<tr>
<td>Serum</td>
<td>Human</td>
<td>59.4</td>
</tr>
<tr>
<td>Prostate</td>
<td>Human</td>
<td>77.8</td>
</tr>
</tbody>
</table>

Table 5.3. Lycopene isomer distribution in tomatoes, related-products and biological samples.
Furthermore, if some *cis* isomers did form during that period, the fact that these isomers were absorbed by the cells is an important finding in itself while lending support to the suggestion that higher solubility of the *cis* forms contribute to higher levels inside the cells. Ultimately, the findings from this study provide conclusive evidence to design future studies examining lycopene's stability *in vivo*.
5.7 References


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