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Measurments of Carotenoid Levels in Human Serum and a Catalog of the Lutein Conformation Populations from Semi-empirical Calculations

Vanesa Mendez
Florida International University, vmend008@fiu.edu

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FLORIDA INTERNATIONAL UNIVERSITY

Miami, Florida

MEASUREMENT OF CAROTENOID LEVELS IN HUMAN SERUM AND A CATALOG OF THE LUTEIN CONFORMATION POPULATIONS FROM SEMI-EMPIRICAL CALCULATIONS

A thesis submitted in partial fulfillment of the

requirements for the degree of

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in

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by

VANESA MENDEZ

2011

To: Dean Kenneth G. Furton
College of Arts and Sciences

This thesis, written by Vanesa Mendez, and entitled Measurments of Carotenoid Levels in Human Serum and a Catalog of the Lutein Conformation Populations from Semi-empirical Calculations, having been approved in respect to style and intellectual content, is referred to you for judgment.

We have read this thesis and recommend that it be approved.

_____
David Chatfield

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Konstantinos Kavallieratos

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John T. Landrum, Major Professor

Date of Defense: October 27, 2011

The thesis of Vanesa Mendez is approved.

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Dean Kenneth G. Furton
College of Arts and Sciences

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Dean Lakshmi N. Reddi
University Graduate School

Florida International University, 2011

DEDICATION

A mis padres.

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ABSTRACT OF THE THESIS

MEASUREMENT OF CAROTENOID LEVELS IN HUMAN SERUM AND A CATALOG OF THE LUTEIN CONFORMATION POPULATIONS FROM SEMI-EMPIRICAL CALCULATIONS

by

Vanesa Mendez

Florida International University, 2011

Miami, Florida

Professor John T. Landrum, Major Professor

Lutein is a principal constituent of the human macular pigment. This study is composed of two projects. The first studies the conformational geometries of lutein and its potential adaptability in biological systems. The second is a study of the response of human subjects to lutein supplements.

Using semi-empirical parametric method 3 (PM3) and density functional theory with the B3LYP/6-31G* basis set, the relative energies of s-*cis* conformers of lutein were determined. All 512 s-*cis* conformers were calculated with PM3. A smaller, representative group was also studied using density functional theory. PM3 results were correlated systematically to B3LYP values and this enables the results to be calibrated. The relative energies of the conformers range from 1-30 kcal/mole, and many are dynamically accessible at normal temperatures.

Four commercial formulations containing lutein were studied. The serum and macular pigment (MP) responses of human subjects to these lutein supplements with doses of 9 or 20 mg/day were measured, relative to a placebo, over a six month period. In each instance, lutein levels in serum increased and correlated with MP increases. The results demonstrate that responses are significantly dependent upon formulation and that components other than lutein have an important influence serum response.

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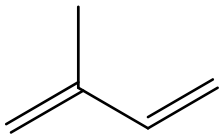
CHAPTER 1: INTRODUCTION

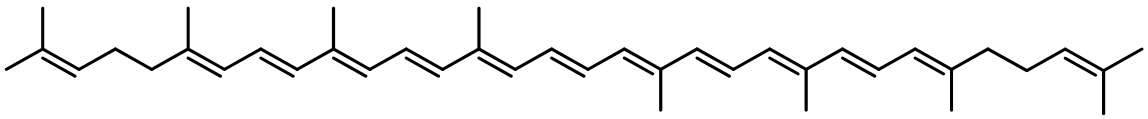
Carotenoids are pigments that are found in plants and microorganisms, but cannot be synthesized by animals [1]. These compounds are considered essential for life since they actively participate in the process of photosynthesis. They are responsible for the coloration in many flowers, fruits and vegetables. Carotenoids are also the principle pigments responsible for color in various animals: fish, birds' feathers and crustaceans [2]. Although more than 750 of these compounds are found in the nature and they are consumed regularly in the human diet only about 20 are detectable in human plasma and tissues [1].

1.1 Carotenoid Structures

Most carotenoids are tetraterpenoids and contain 8 isoprenoid units [3], Figure 1. Thus they have a C_{40} carbon skeleton. The carotenoid is built up by the linking of isoprenoid units in a head to tail pattern to form two C_{20} fragments that are linked together in a head to head fashion at the center. Consequently, at the center the two methyl groups of the polyene chain are six carbons apart from each other but they are only 5 carbons apart in other rest of the molecules.



Isoprene unit



C_{40} Structure of Lycopene

Figure 1. Carotenoid structure

C_{40} carotenoids are derived from lycopene by different synthetic pathways: cyclization, insertion of oxygen, double bond migration, methyl migration, chain elongation, and chain shortening [3].

Traditionally, carotenoids can be grouped into two classes: (a) carotenes or (b) xanthophylls. Carotenes are hydrocarbons (example: lycopene or β-carotene). Xanthophylls have oxygen atoms in their structure (in example: lutein and zeaxanthin).

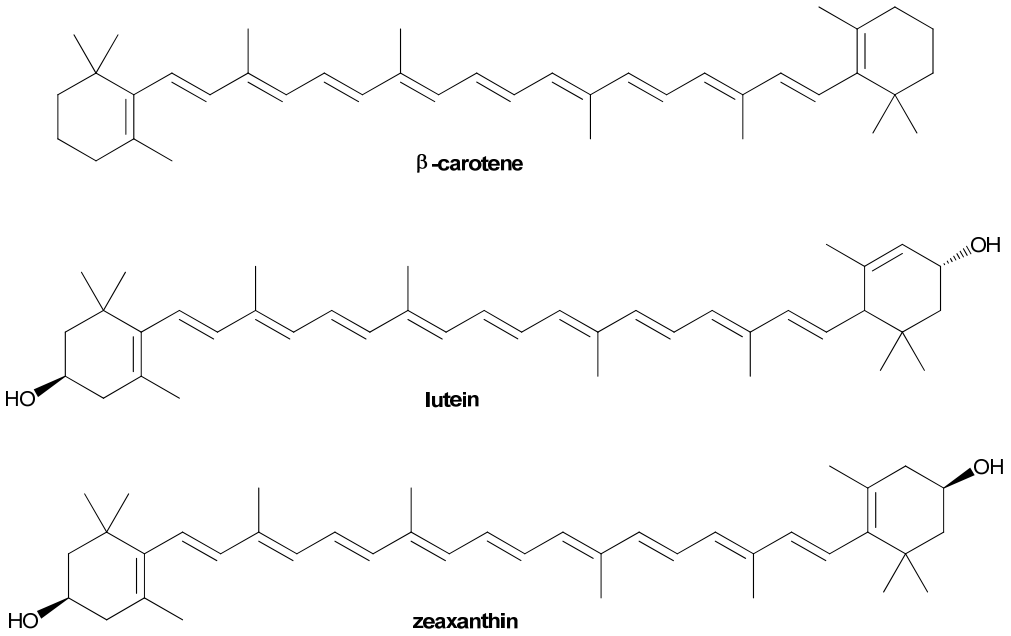


Figure 2. Carotene and Xanthophylls

For carotenes, all specific names are based on the stem name "carotene", which corresponds to the structure and numbering shown in Figure 3.

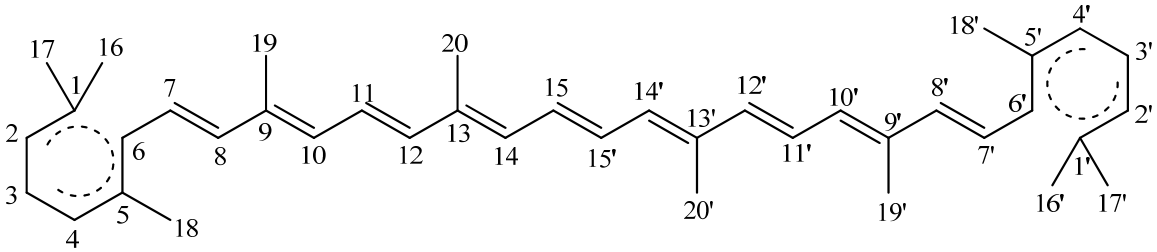


Figure 3. Carotene numbering system

1.2 Carotenoid End-Groups

Structurally, the carotenoids possess end-groups that are either acyclic or a ring of five or six carbons at one or both ends of the molecule. Carotenes are named according to the end group(s) that they contain in their structures[3]. The end-groups and their prefixes are indicated in Table 1.

Table 1: End-Group Designations of Carotenes.

Type	Prefix	Structure
Acyclic	ψ	(a)
Cyclohexene	β, ε	(b, c)
Methylenecyclohexane	δ	(d)
Cyclopentene	κ	(e)
Aryl	ϕ, χ	(f,g)

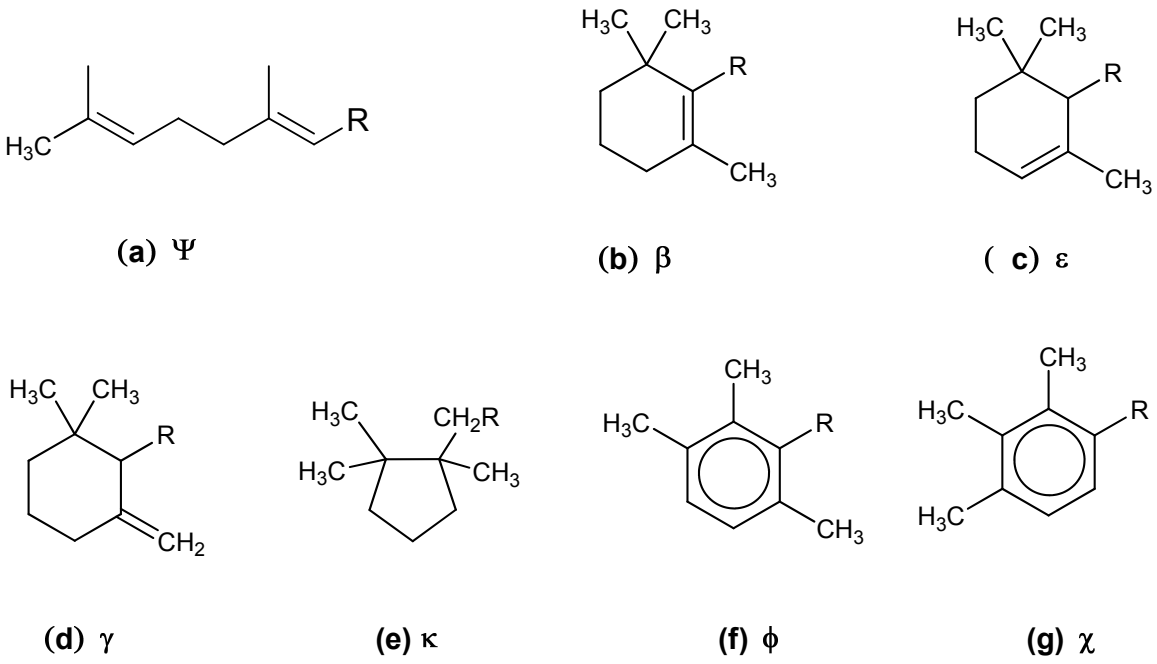


Figure 4.End-groups structure

In green tissues, algae, and many fungi the major carotenoids are cyclic compounds such as: β-carotene and α-carotene. The mentioned carotenes contain ionone rings in their structure. β-carotene has two β-ionone groups and α-carotene has one ε -ionone and one β-ionone rings.
In xanthophylls, which contain oxygen functional groups, the compounds are named according to the usual IUPAC nomenclature rules, indicating the position of the oxygen containing substituent. For example, the name of zeaxanthin (see Figure 2) is β,β -carotene-3,3'-diol.

1.3 Biosynthesis [3, 4]

In plants, carotenoids are synthesized in the plastids by enzymes that are encoded by the nucleus. Carotenoids are built from the biological isoprene precursor isopentenyl pyrophosphate (IPP). The first specific terpenoid precursor is mevalonic acid (MVA). Mevalonic acid is converted into IPP by means of a three step enzymatic pathway; each step requires one mole of ATP per substrate. The first step is regulated by MVA kinase and this has been demonstrated in plant species and microorganisms. The identity of MVA-5-pyrophosphate, the product resulting from the second step, has been evidenced in yeast and has been identified as an intermediate in a bacterial system that can convert MVA into carotenes. Isopentenyl pyrophosphate can be found in enzymatic systems capable of carotenoid synthesis, like in tomato fruit.

To continue the chain elongation, in the first step IPP is isomerized to dimethylallyl diphosphate (DMAPP) by the enzyme IPP isomerase [5] (See Figure 6). The sequential addition of three IPP molecules to DMAPP, lengthens the chain to 10 carbons. Formation of the C_{15} molecular intermediate (FPP) is catalyzed by the enzyme geranylgeranyl diphosphate synthase (GGPS) that links DMAPP. The enzyme is one member of a closely related family of prenyl

transferase enzymes that are distinguished by the length of the final product. The 20-carbon molecule geranyl geranyl pyrophosphate (GGPP) is generated by two identical C_5 addition steps.

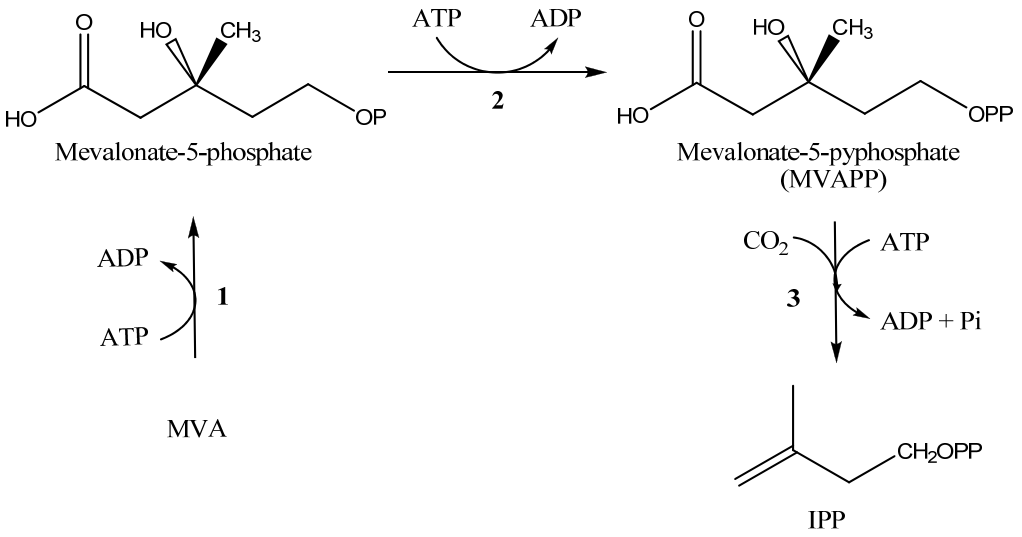


Figure 5. Formation of IPP from MVA

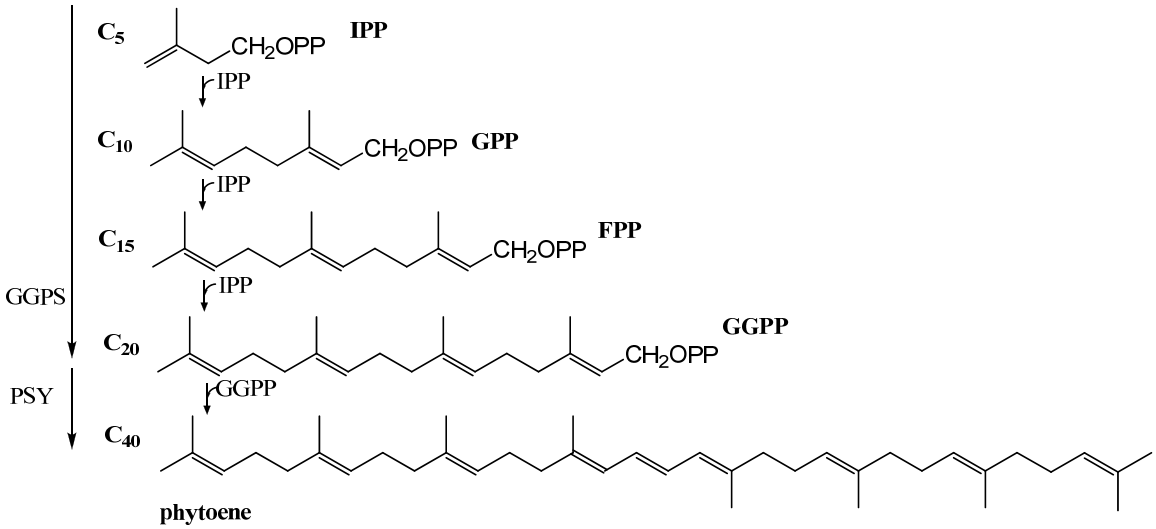


Figure 6. General pathway for the formation of phytoene [4]. The arrows on the side represent the enzymes involved. GGPS: geranylgeranyl diphosphate synthase. PSY: phytoene synthase.

The next step in the carotenoid pathway is the condensation of two GGPP molecules to produce phytoene, which is catalyzed by a membrane-associated enzyme, phytoene synthase (PSY). Phytoene synthase shares amino-acid sequence similarity with GGPP synthase and other prenyl-transferases [5]. The condensation of two molecules of farnesyl pyrophosphate to produce squalene, the C_{30} precursor of sterols, is similar to the formation of phytoene from geranylgeranyl diphosphate synthase. In this biosynthesis pathway, NADPH is not required and one hydrogen is lost from C1 of each of the participating GGPP molecules. The GG residues are linked via sulphonium ylide.

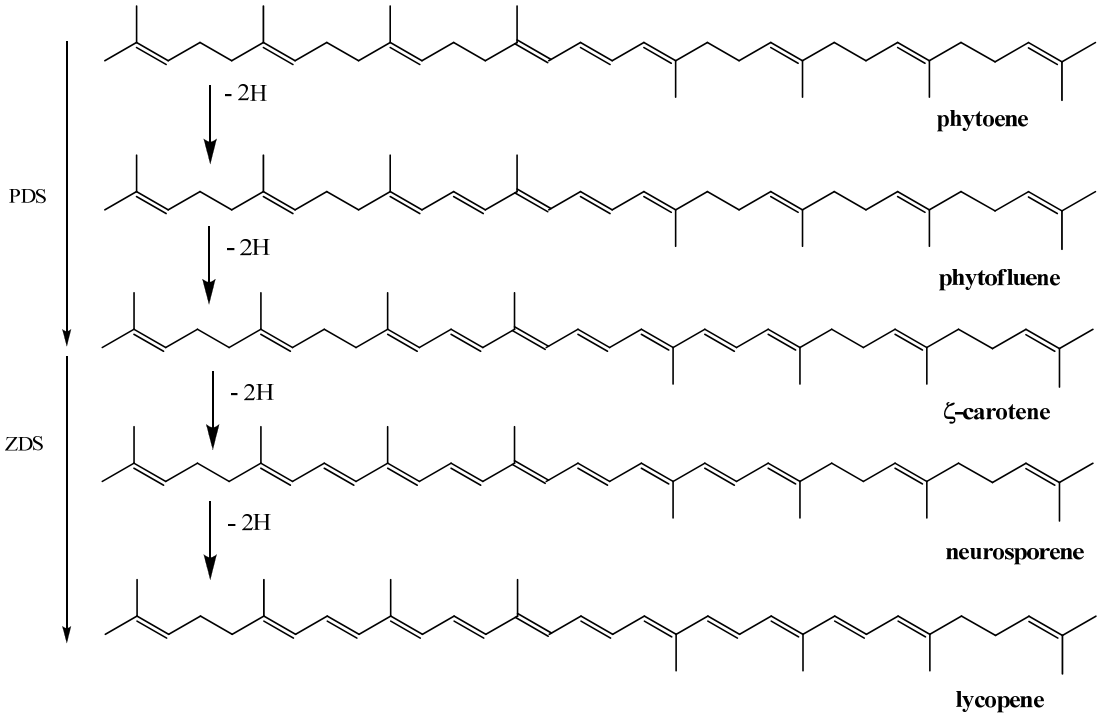


Figure 7. General pathway for the formation of lycopene [4]. The arrows on the side represent the enzymes involved. PDS: phytoene desaturase. ZDS: ζ-carotene desaturase.

Four desaturation steps are required for the conversion of phytoene to lycopene and are catalyzed by two related enzymes in plants: phytoene desaturase and ζ-carotene desaturase.

However, bacteria and fungi achieve the same result via a single enzymatic complex. The first desaturation of phytoene produces phytofluene; then phytofluene undergoes dehydrogenation and isomeration to be converted into ζ-carotene. ζ-carotene is subsequently desaturated to produce neurosporene and lycopene. In the end the four reactions build the conjugated carbon backbone of lycopene.

At this point in the biosynthesis, the pathway branches and the cyclization of lycopene may occur to produce either β– or ε-ring end-group structures. One branch will lead to the formation of β-carotene and its derivative xanthophylls, and the other branch leads to α-carotene and lutein. ε-carotene is also produced from δ-carotene.

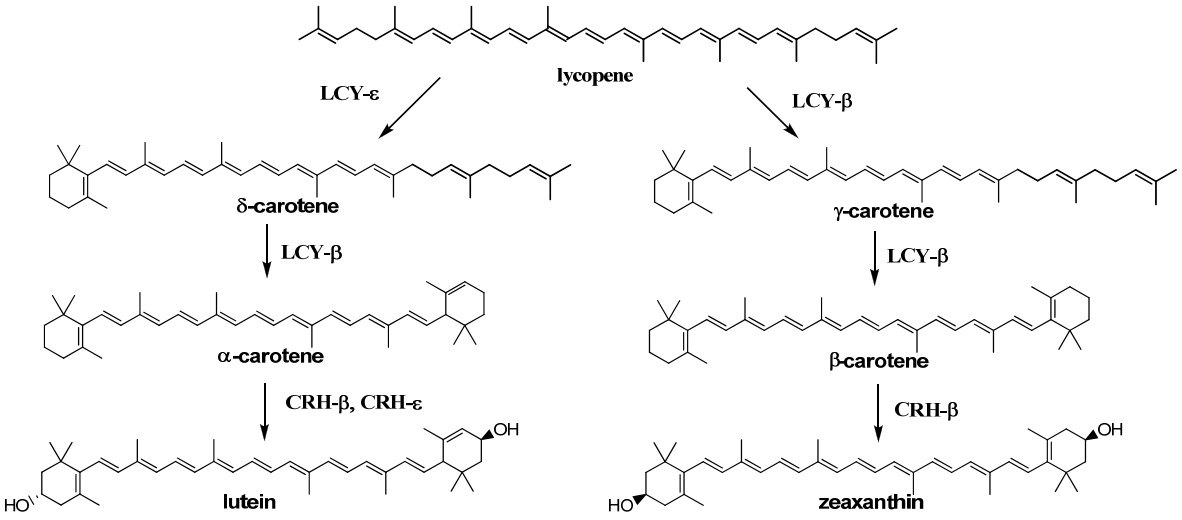


Figure 8. Cyclization of lycopene. Enzymatic conversions are shown by arrows with the enzymes responsible. LCY-β: lycopene beta-cyclase; LCY-ε, lycopene epsilon-cyclase; CRH-β, β-carotene hydroxylase; CRH-ε, epsilon-carotene hydroxylase [5].

During the biosynthesis of the cyclic carotenoids, the formation of ε- and β-ionone rings take place at a late stage in the pathway. Experiments indicate that the β-ionone ring is formed by

a mechanism that involves a carbonium ion (Figure 9). The cyclization concludes with the release of the H_B. proton from the C-6. ε-ionone is also formed by via a carbonium ion but in this case it is stabilized by expulsion of the H_A proton at C-4 [6].

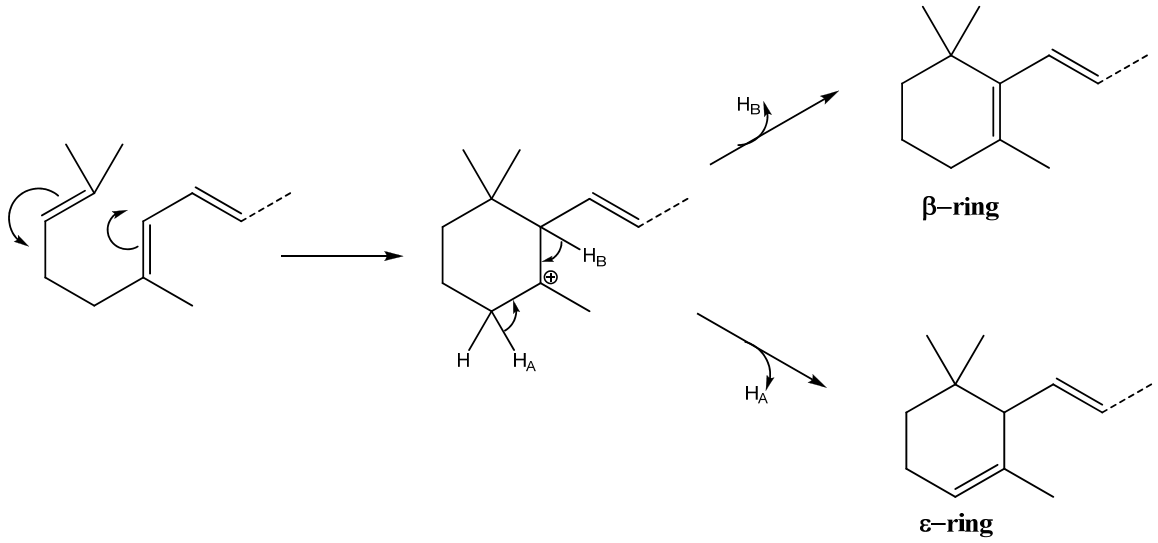


Figure 9. Basic mechanism for biosynthesis of ε and β-rings in carotenoids

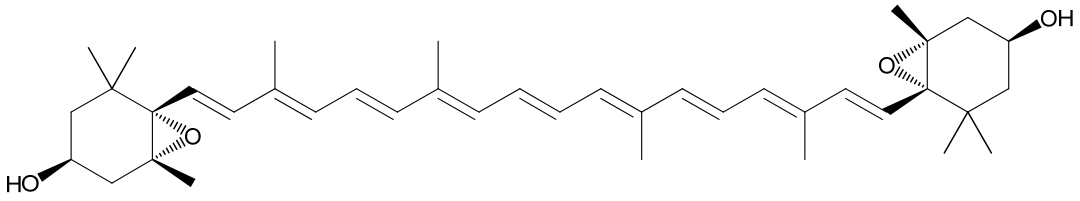
During the cyclization of lycopene, the reactions that are necessary for the formation of the carotenoid end-groups in both branches (the one that leads to lutein and the second that leads to zeaxanthin, Figure 8) are dependent on the relative activities of the cyclases involved in the reaction. The most common xanthophylls in the green tissue of higher plants, algae, and non-photosynthetic bacteria have hydroxyl substitutents at C-3, an oxo at C-4 or an epoxy at the 5, 6 position of the ionone ring [7]. Genetic evidence and functional analysis of an Arabidopsis β-hydroxylase enzyme support the existence of separate hydroxylases specific for the β- and ε-rings.

1.4 Cis-carotenoids [8]

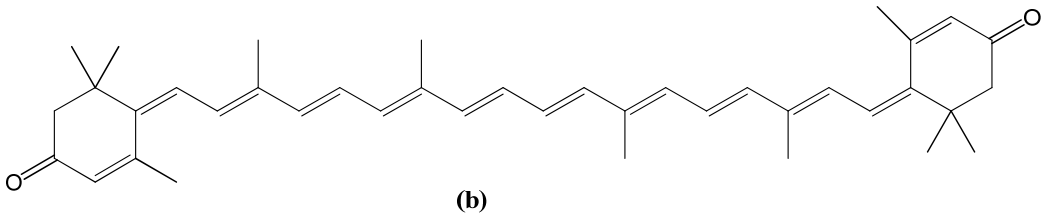
The conjugated double bonds present in the structures of carotenoids give them the possibility to exist as several "*cis-trans"* geometrical isomers. Usually, we use c*is* and *trans* to indicate the orientation of substituent groups in a molecule relative to a double bond. When we have more than two different substituents on a double bond, *E* and *Z* are the more correct designations used to describe this geometrical isomerism. In nature, most carotenoids are found in the *all-E* (*trans*) configuration. It is also possible for the structures to have one or more than one "*cis" or Z* double bond, but these are not energetically favored.

The β-carotene structure was elucidated by Karrer in 1930. In 1936, Gillam and El Ridi [9] reported in their study that when β-carotene was absorbed in an aluminium oxide column, it separates into two zones with different absorption maxima. They attribute this phenomenon to isomerization of β-carotene. With those studies the authors opened an unknown area in carotenoid research and from there more studies on *cis* and *trans* carotenoids were carried out. Further studies reported that in carotenoids other than β-carotene the same behavior was observed and more experimental data were presented to justify the conclusion that *cis-trans* isomerization was observed in all carotenoids.

As stated above, many geometrical isomers can potentially arise from a single molecule of a carotenoid. The number of stereoisomers in an "unsymmetrical" carotenoid (like lutein) with *"n"* double bonds rises to 2^n possibilities. For "symmetrical" carotenoids, we have two cases: the first one, when *"n"* is odd, the number of stereoisomers is $2^{(n-1)/2}(2^{(n-1)} + 1)$, for example β-carotene or violaxanthin. The second one, when *"n"* is even, the number of stereoisomers is $(2^{(n/2)} - 1)\ (2^{(n/2)-1)} + 1)$, for example rodhoxanthin.



(a)



(b)

Figure 10. Examples of symmetrical carotenoids: (a) violaxanthin, (b) rhodoxanthin.

In practice, there is a difference between the number of theoretically calculated and experimentally observed steroisomers. Experiments show that each carotenoid, after treatment to produce a mixture of isomers, produces only a limited number of *cis* isomers. The outcome of this reaction can be attributed to the relative energies of the *all-trans (E) isomers* compared to those in which a *cis* (Z) geometry is present at some point in the polyene chain. The other important factor that must be considered is that proposed by Pauling. Pauling noted that isomerization can be favored as a result of overlapping of the methyl group of a carbon atom adjacent to a double bond and the hydrogen. We can explain this concept for carotenoids by looking at the backbone chain structure of the carotenoid, if we picture its orientation as seen in Figure 11:

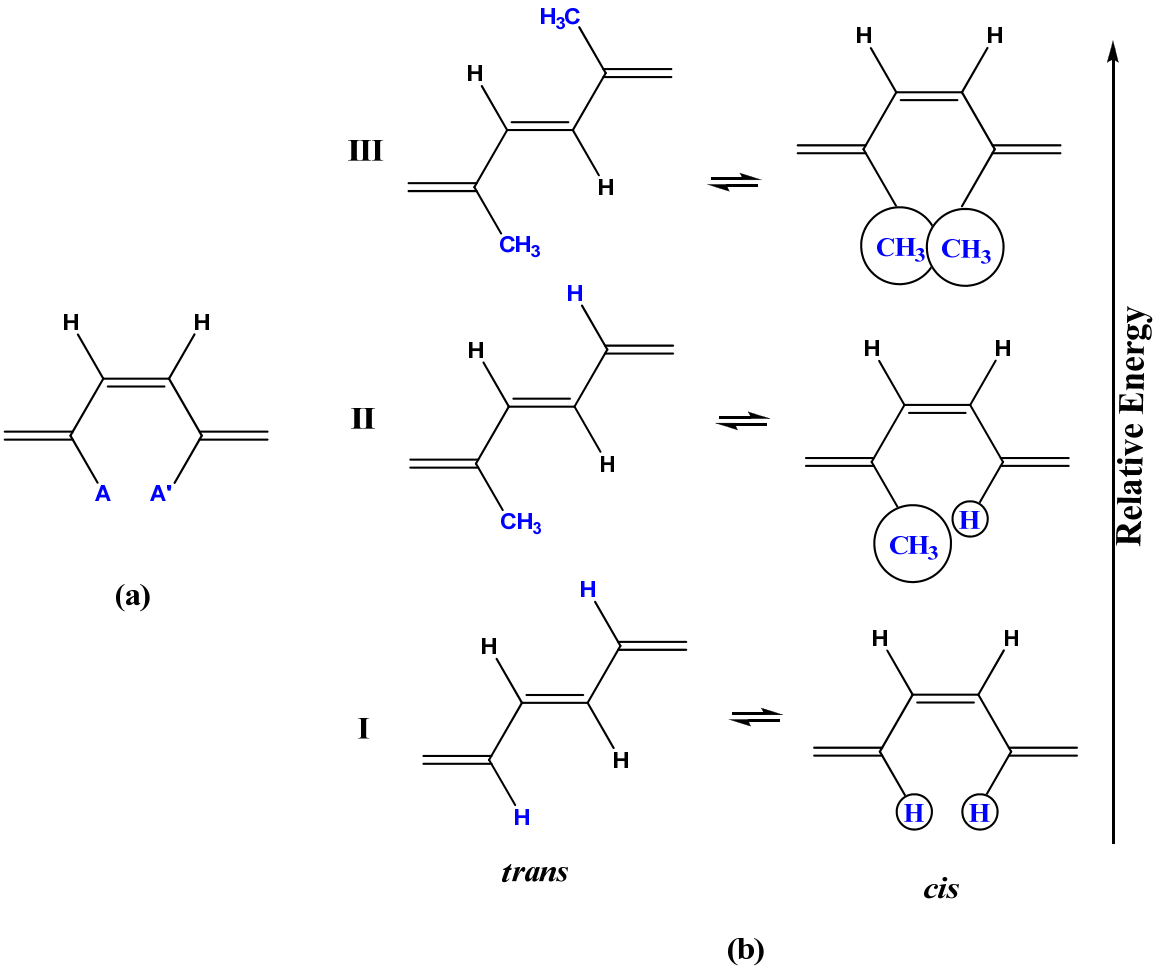


Figure 11. (a) Representation of a *cis* double bond in the polyene chain. (b) *trans-cis* cases

In this system A and A' may represent two hydrogen atoms, two methyl groups or one of each. If both A and A' are hydrogens (case I, Figure 11.b) the rotation around the central double bond will not meet any significant steric barrier in the planar *cis* geometry. However, if A is a methyl group and A' is and hydrogen (case II, Figure 11.b) or another methyl group (case III, Figure 11.b) the *cis* geometry would have to overcome a larger energy barrier to exist.

Isomerization from *trans* to *cis* often occurs during chemical reactions, via a thermal or a light activated pathway, but may it also occur via reaction with an enzyme. Geometrical isomerization leads to a change of shape in the carotenoid molecule. The molecule shows bending

and can form a zig-zag structure, see Figure 12. Presumably these shapes might have an important role in reactions with enzymes or with other proteins within the tissues.

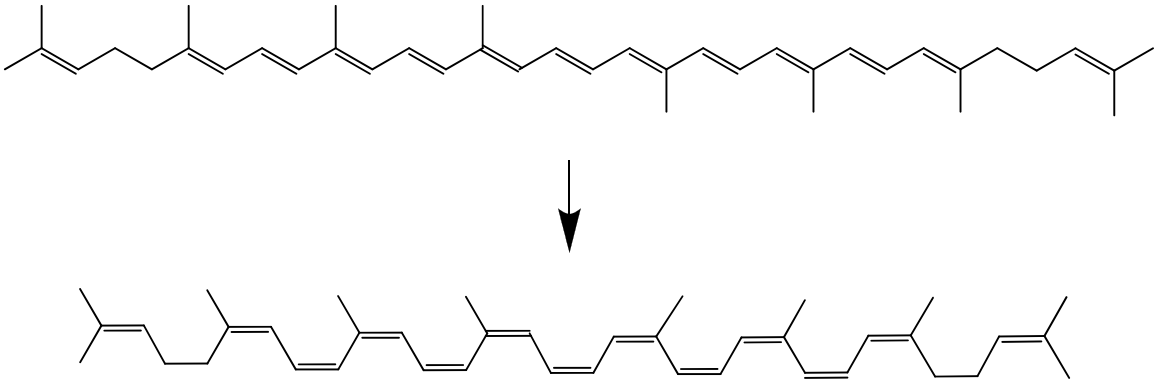


Figure 12. *cis* isomer of Lycopene

1.5 Properties of cis carotenoids

The following are some properties observed experimentally in *cis* isomers. The relative stability of an all-*trans* (*E*) carotenoid is greater than that of *cis* isomers, because of the better electron delocalization and the more planar nature of the chromophore configuration.

According to Zechmeister's study; in an all-*trans* carotenoid solution, the carotenoid undergoes isomerization continually, first to mono*cis*, next to di*cis,* and so on, during that process the structure star bending until a poly*cis* solution is formed. The thermostability of the carotenoid solution first decreases during the isomerization, and during the process it gets to a point where the molecule is completely bent, because of its poly*cis* configuration. In the end the structure accommodates in a compact overall shape so that the thermostability increases again.

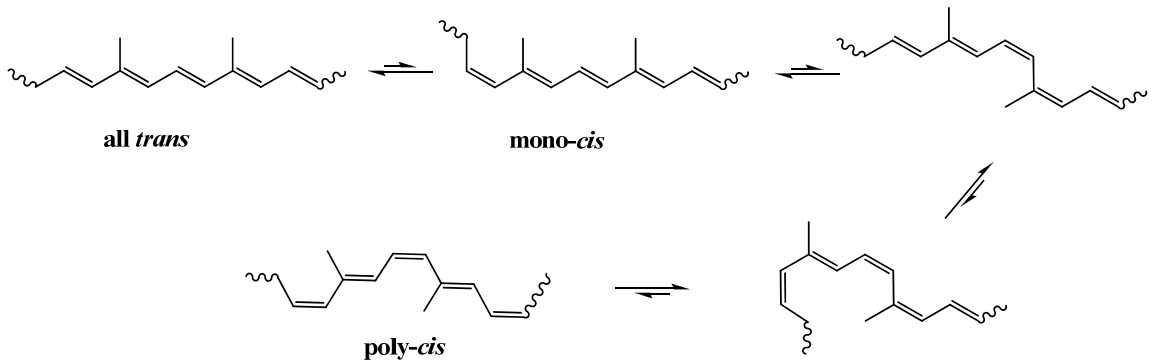


Figure 13. Scheme of isomerization of carotenoids.

Cis carotenoids are photochemically sensitive in the presence of iodine. Studies have shown that the all-*trans* isomer has the highest melting point of all the isomers. By comparison the *cis* conformers melt at lower temperatures. Interestingly, the decreasing of the melting point in a set of isomers is not proportional to the number of the *cis* bonds present in the molecule.

Spectral characteristics of all-*trans* carotenoids and mixtures of *cis*-*trans* carotenoids were analyzed by Zechmeister and Polgar [10]. Natural or synthetic carotenoids were treated by reflux for 45 minutes. The product was compared with an identical sample fraction that was treated with iodine for an hour at room temperature. It was observed that the color intensity decreased for the mixture of *cis*-*trans* isomers. The height and wavelength of the visible and ultraviolet maxima decreased in each case (Figure 14).

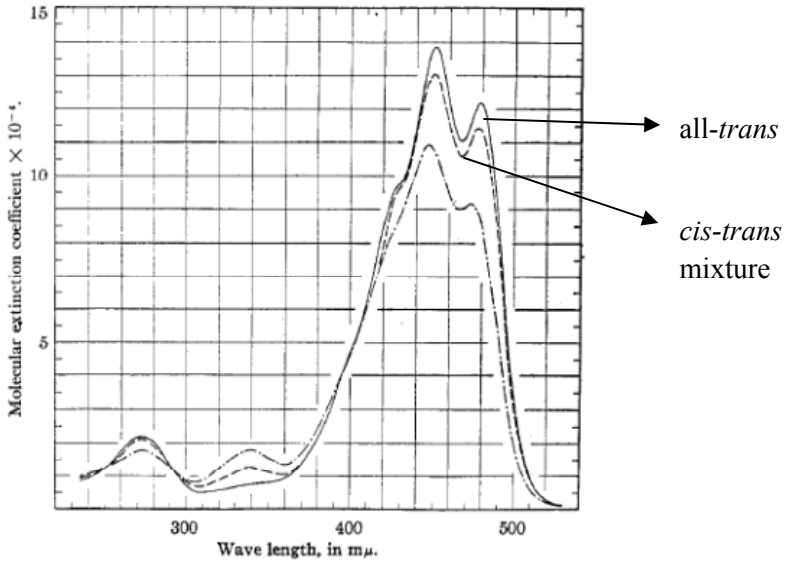


Figure 14. Molar extinction curves of β-carotene in hexane: ___ *all-trans* solution. --- mixture of *cis*-*trans* isomers after refluxing. _._._mixture after iodine treatment [10].

1.6 Naturally occurring *cis* carotenoid isomers.

There are some carotenoids that have been identified in nature only as *Z* isomers. Bixin was the first *cis*-polyene to be recognized in nature. It is isolated from annatto seeds (*Bixa Orellana*). Pro-lycopene was first detected to exist in the tangerine tomato in 194. The tangerine tomato has an orange color and accumulates pro-lycopene (7Z, 9Z, 7'Z, 9'Z-tetra*cis*-lycopene) instead of the all-*E*-lycopene that is found in most tomatoes. Photoisomerization of pro-lycopene occurs in chloroplasts and is associated with the existence of the photosynthetic apparatus[11]. Studies have focused on trying to find a particular enzyme and/or look for possible environmental factors that can affect the isomerization of carotenoids in plants [11],[12]. But despite all of these efforts to understand the mechanism of carotenoid isomerization, it remains unclear how they are produced. Isomeric forms of lycopene can exist as the 5-*cis*, 9-*cis*, 13-*cis* and 15-*cis* isomers [13].

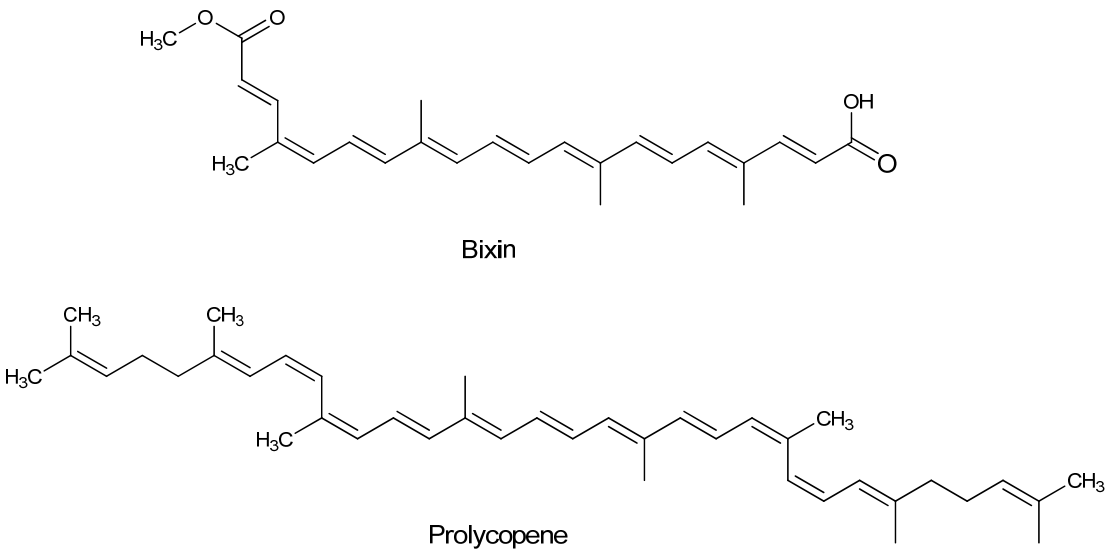


Figure 15. Natural *cis* carotenoids.

1.7 Bioavailability of *cis*-carotenoids [14]

Isomerization produces a significant difference in molecular structures that can affect the bioavailability of carotenoids. In tissues, approximately equal amounts of all-*E* and various *Z*-lycopene isomers can be found. The *Z* isomers have a greater solubility in mixed micelles and are less likely to aggregate or crystallize; as a result they more easily become incorporated into bile acid micelles, increasing the efficiency of their transport to tissues.

In fruits and vegetables β-carotene is found in the all-*E* form and only small amounts of the *Z* isomers are detected. The same is true for carotenoids in human plasma, but in some tissues *Z*-β-carotene isomers accumulate to significant levels. These observations may be to the result of various factors such as a differential uptake of *cis* isomers, as well as differences in their absorption from the gut and transport in the human blood stream. Studies in gerbils after including β-carotene and vitamin A in the diet showed that the amounts of (*9Z*) - and (*13Z*)- β-carotene found in the liver were nearly equal to the amount of all-*E* β-carotene. An *in vitro* model of digestion showed that the incorporation of *Z* isomers of β-carotene into micelles were 2-3

times more efficient than the *E* form. Lutein and zeaxanthin have been found in their *cis* isomers form: (*13 Z*)- and (*13'Z*)- lutein isomers; (*9Z)*- and (*9'Z*)- lutein; and *(13-Z)*- zeaxanthin in processed fruits, vegetables and pasta food products [15]. It has been demonstrated that *trans-cis* isomerization is an effect of the thermal processing of vegetables and fruits [16]. Studies [17, 18] have isolated the previous mentioned *cis* isomers of lutein and zeaxanthin (including 15*Z*-zeaxanthin) in human serum and milk. Although the quantities isolated are lower in comparison with the all-*E* isomers, it is shown that those xanthophylls isomers also exist in the human tissues.

1.8 Macular Pigment Carotenoids (MPC)

Lutein and zeaxanthin are two yellow carotenoids that give the macular area of the retina its color. The reason they are known as macular pigment carotenoids is that they are selectively accumulated in this part of the eye. The concentration of the macular pigment in the central retina approaches 1 mM, more than 1000 times that in human serum and liver[19].

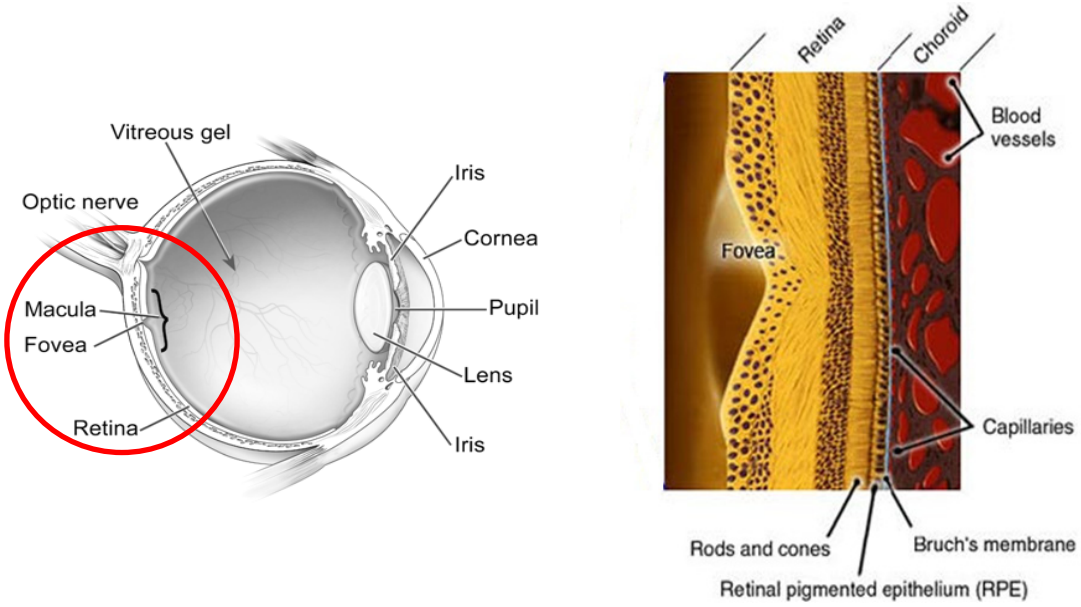


Figure 16. Representation of the human retina.

(From: http://www.gene.com/gene/products/education/tgr/eye-health-glossary.html)

The difference between these two molecules that compose the macular pigment is the location of one double bond in one of the end-groups and also the configuration of the hydroxyl group in that particular ring. Bone and Landrum showed that the major components of the macular pigment were lutein [(3R,3'R,6'R)- β,ε-Carotene-3,3'-diol], zeaxanthin [(3R,3'R)- β,β-Carotene-3,3'-diol] and meso-zeaxanthin [(3R,3'S)- β,β-Carotene-3,3'-diol][20]. Neither humans nor animals can synthesize carotenoids, so they must obtain them exclusively from their diet. Green leafy vegetables, fruits, and egg yolk are the primary sources of lutein and zeaxanthin available in nature.

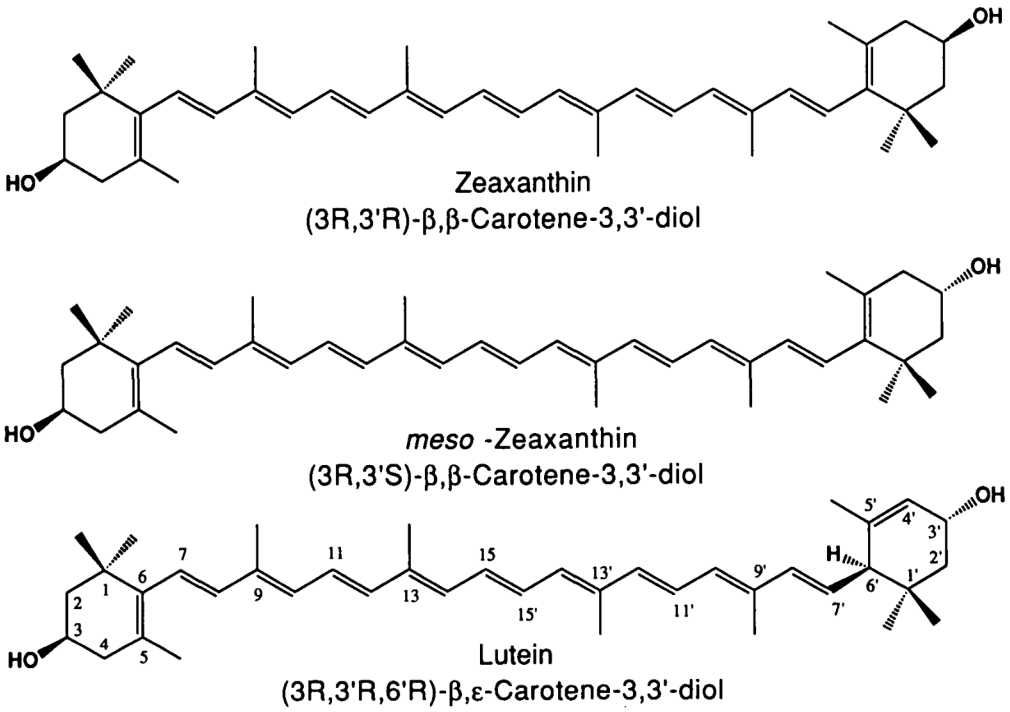


Figure 17. Major components of the macular pigment [20]

The content of lutein, zeaxanthin, and meso-zeaxanthin in the retina is approximately 36%, 18%, and 18% respectively of the total carotenoid in the retina[21]. These carotenoids are

concentrated in the fovea, which is the depression located in the retina coinciding with the position of maximum visual acuity.

Lutein and zeaxanthin can be characterized by their UV-visible spectra (Figure 18). The spectra may seem very similar but the presence of two β-ionone rings at the ends of the of the zeaxanthin structure extend the conjugation of the polyene chain and lower the energy separation between the ground and excited state, shifting its spectrum slightly to the red. Lutein absorbs at a slightly shorter wavelength than zeaxanthin.

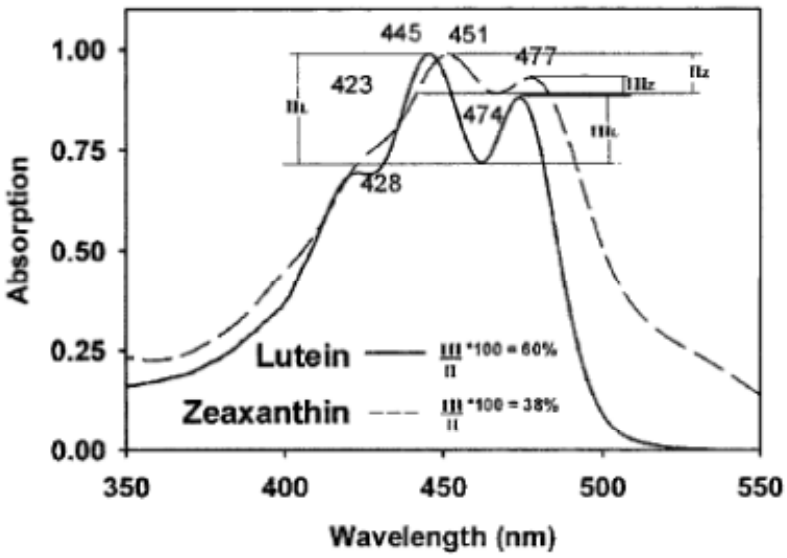


Figure 18. Spectra of lutein and zeaxanthin in ethanol[21].

On the basis of the spectra we can state that both carotenoids can filter blue light effectively. The retina is susceptible to oxidative stress because of the high demands for oxygen and its constant expose to light. A principal function of lutein and zeaxanthin as macular pigments appears to be the protection of the retina against photo-induced damage by acting as antioxidants in addition to their role as a filter for blue light.

1.9 Transport and distribution of MPC

The absorption of lutein and zeaxanthin occurs in the enterocytes (intestinal absorptive cells) of the intestinal mucosa. They enter the hepatic portal circulation in the form of chylomicrons [22]. In the liver, low and high density lipoproteins (LDL, HDL) are synthesized and transport lutein and zeaxanthin to other tissues. It has been demonstrated that HDL is the primary carrier of lutein and zeaxanthin and that LDL is principally responsible for the transportation of carotenes[23]. Dietary lutein may be metabolized to produce meso-zeaxanthin within the retina, although the exact mechanism is still unknown[20]. A clear pathway of how these carotenoids are transported to the retina is still not well-defined. Li et al.[24] proposed a possible pathway for the uptake, transport and accumulation of these macular pigments carotenoids in the retina based on their studies on carotenoid-binding proteins. They suggest that the uptake of carotenoids within the cholesterol containing lipoproteins is mediated by HDL and HDL receptors, such as SR-BI (a cell surface glycoprotein that binds HDL). The mechanism would result in the delivery of lutein and zeaxanthin to the retinal pigment epithelium (RPE). From the RPE a pathway analogous to the transport of retinoids within the retina is suggested for lutein and zeaxanthin. Accumulation of the MPC in the retina may be similar to a pathway known to deliver lutein to the silk glands in silkworms. In these larval insects, lutein is delivered to the silk gland via a pathway involving specific cell-uptake and specific binding proteins.

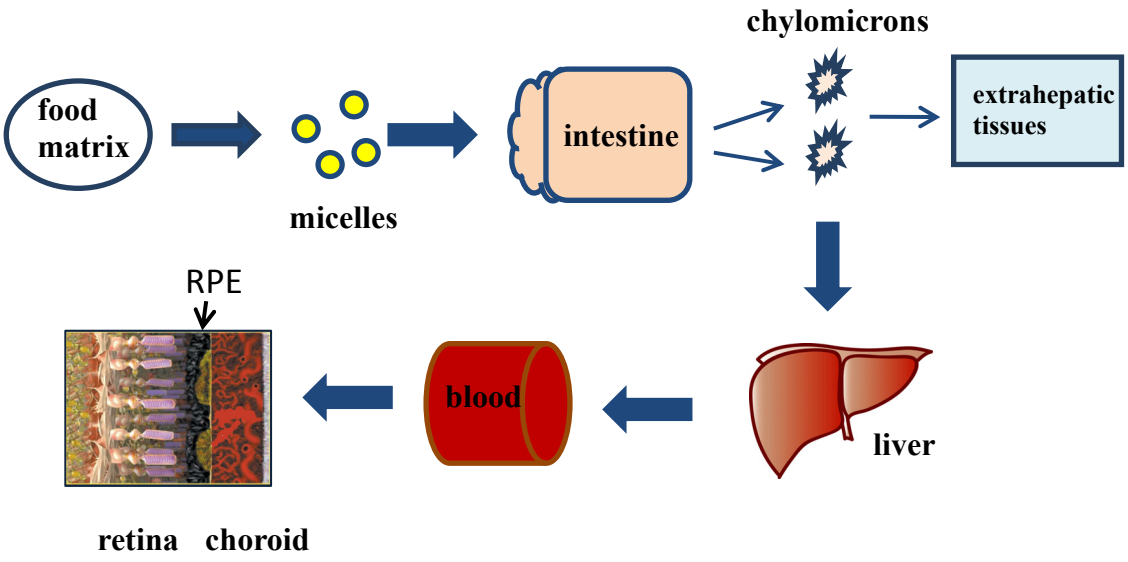


Figure 19. Scheme of possible pathway for the uptake, transport, and accumulation of the macular carotenoids in the human retina. RPE (retinal pigment epithelium).

As a general pathway for absorption and metabolism of carotenoids in animal models [25], it is known that after the consumption of carotenoids from the daily diet, these are incorporated in a complex food matrix with proteins and lipids, because of their lipophilicity. After the food matrix is digested, carotenoids will be combined with lipids and bile salts to form micelles. The micelles will move to the intestinal brush border membrane, and carotenoids will be captured and transported into the enterocyte to be metabolized and secreted into the intestinal lumen (cavity where the nutrients are absorbed) where they will be incorporated to chylomicrons and secreted into the lymph. Chylomicrons take the carotenoids to the liver where they can be processed by the liver and stored there or resecreted in VLDL (very low-low density-lipoprotein) and then to the bloodstream in the form of LDL and HDL. Carotenoids can also be captured by extra-hepatic tissues before they are taken to the liver. Lutein and zeaxanthin will be transported with HDL in the bloodstream, and the uptake of lutein and zeaxanthin on RPE cells will be mediated by the SR-B1 (scavenger receptor class B member 1, is a receptor for HDL) [24]. The

selection of these carotenoids is probably determined by the selective binding proteins GSTP1 [24] (glutathione s-transferase P1) and StARD3 (steroidogenic acute regulatory domain protein 3) to mediate this process [26]. Experiments show that human GSTP1 exhibits a high affinity for zeaxanthin, and human StARD3 proteins bind lutein. Recently Bernstein's research group identify StARD3 as a lutein-binding protein in the macula of the primate retina[26]. Antibody labeling of retina cross-sectioned reveals that these proteins are found distributed in the same structures as the macular pigment.

1.10 Age-Related Macular Degeneration (AMD)

Age-Related Macular Degeneration is a disease that affects the central visual acuity. It is associated with age and is the leading cause of blindness in people over 65 years old [27]. Age-Related Macular Degeneration is associated with abnormalities in the retina, degeneration of the RPE, and ultimately loss of the photoreceptors. Low levels of macular pigments appear to be a risk factor for AMD [28]. Studies report that high carotenoid intake is associated with a 43% risk reduction for exudative AMD. Lutein and zeaxanthin have been shown to be the most strongly associated, among all the carotenoids studied, capable of reducing the risk for AMD [29]. The AREDS N°22 reported that participants with highest dietary intake of lutein and zeaxanthin were half as likely to have an advanced AMD as those with the lowest intake [30].

One of the principle reasons for our study of lutein and zeaxanthin is the properties that enable them to reduce the risk of AMD. A central characteristic of MPC is their ability to filter blue light. Blue light strikes the human retina and causes photooxidative damage to the lipid components of the photoreceptors disk membranes, which is a demonstrated characteristic of the pathology of AMD[21].

1.11 Principal properties of carotenoids [31, 32]

As we mentioned above, carotenoids have an important role protecting cells and organisms against damage by light and reactive oxygen species (ROS) generated by light. The presence of conjugated double bonds in the structure of carotenoids is responsible for their most distinctive characteristic, light absorption [33].

Carotenoids can quench or inactivate the excited states of molecules. Krinsky explained how carotenoids can perform as antioxidants by reacting with active oxygen species in photobiological systems [32].

Carotenoid quenching of singlet molecular oxygen ($^1O_2$)

The molecule O_2 is required for all aerobic cell metabolism in nature. However, the excited state form of O_2 singlet oxygen ($^1O_2$), is an unstable and highly reactive molecule particularly toward many biological substrates. The unstable form of oxygen can react with phospholipids compounds, that can be found in the biological cells [34]. Cell damage is frequently mediated by the formation of $^1O_2$ in photosensitized reactions. To explain how it is formed, it is helpful to understand the different states in the O_2 molecule. In the ground-state, O_2 has two electrons in two separate π orbitals with their angular momentum opposed but with parallel spins. This ground-state is designated as the $^3\Sigma$ because O_2 exists as a triplet molecule and is a diradical. In the lowest excited state, $^1\Delta$, the two electrons share a single orbital and they have the same spin. Since the electrons in this state are paired in accordance with the Pauli principle, the molecule is in a singlet state. O_2 also has a second singlet state, $^1\Sigma$. This state has a lifetime of only $1x10^{-11}$s and decays to the $^1\Delta$ state.

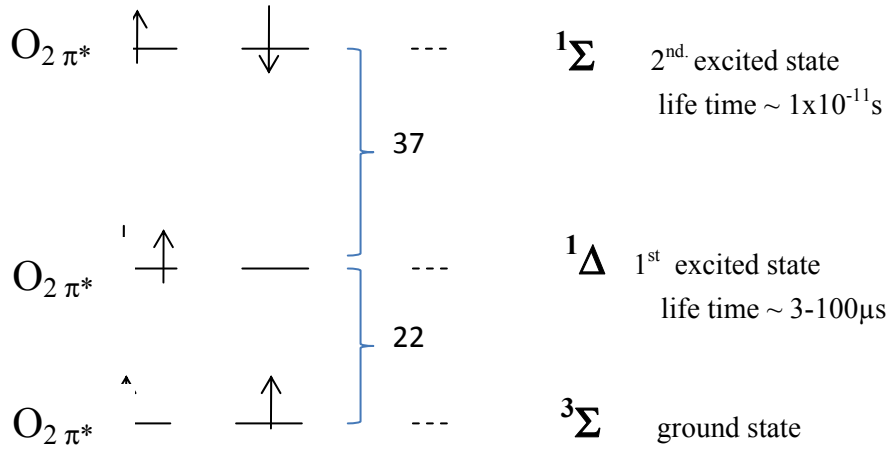


Figure 20. Diagram of the states of the O_2 molecule.

When light strikes a biological system that contains an appropriate sensitizer (S) the energy of the photon can convert the sensitizer to an electronically excited form (^{1}S). The singlet excited states for most molecular species are very short-lived, typically measured in nanoseconds (<10 ns usually). By intersystem-crossing the singlet excited states, ^{1}S, can often be efficiently coverted to a longer lived triplet excited state, ^{3}S (Equation 1).

$$S \xrightarrow{h\nu} {}^1S \longrightarrow {}^3S \qquad (1)$$

The ^{3}S can initiate Norrish Type I or Type II photochemical reactions. In type I reactions the triplet sensitizer initiates radical reactions by abstracting an electron or hydrogen atom from nearby biomolecules creating reactive radical species (Equation 2). In the Type II reaction, the triplet sensitizer reacts with molecular oxygen. Since O_2 is also a triplet species, formation of the highly reactive $^1O_2$ can occur renegerating the ground-state sensitizer (Equation 3), conserving the total spin of the system as required by quantum mechanics.

Type I: ^{3}S + biomolecule $\xrightarrow{RH}$ S^+, SH·, R· (2)

Type II: $^3S + {}^3O_2 \longrightarrow {}^1S + {}^1O_2$ (3)

Ground state carotenoid pi bonds (singlets) interact readily with $^1O_2$, accepting the excess energy from this reactive excited state. In this physical quenching of the oxygen excited state the carotenoid absorbs the energy from the $^1O_2$ and the result is the formation of the triplet state carotenoid (Equation 4). This reaction conforms to quantum mechanical restrictions that forbid a net change of spin state during any allowed process.

$^1\text{CAROTENOID} + {}^1O_2 \longrightarrow {}^3\text{CAROTENOID} + {}^3O_2$ (4)

$^3\text{CAROTENOID} \longrightarrow {}^1\text{CAROTENOID} + \text{heat}$ (5)

The presence of the conjugated polyene chain in the carotenoid enables these molecules to readily dissipate the energy through rotational and vibrational transitions and through collisions with the solvent liberating the excess energy harmlessly as heat. Thus the triplet carotenoid returns to its ground state unaltered (Equation. 5).

As we can see, the carotenoid may act as an antioxidant by neutralizing the potentially harmful $^1O_2$ molecule in tissues and cells. Our research is concentrated on lutein and zeaxanthin and its importance to living organisms.

In an effort to understand the nature of these two molecules in nature, and the uptake and transport of lutein and zeaxanthin in the human body we conducted two studies. The first is a theoretical study. We have cataloged the different possible conformations of the lutein molecule model resulting from *s-cis* geometries in the polyene chain. The goal of this study is to provide a better understanding of the possible interactions of carotenoids within the environment in the cell. The second part of this thesis addresses an applied project in which we studied the bioavailability

of lutein and the relationship of the levels of six major carotenoids in human serum before and during supplementation with lutein.

CHAPTER 2: A CATALOG OF THE RELATIVE ENERGIES OF LUTEIN CONFORMATION POPULATIONS

2.1 Overview:

Carotenoids are characterized by the presence of a polyene chain, typically having 9 conjugated double bonds, predominantly having an all-*E* (or all-*trans*) configuration. These structures can acquire different conformations because of the rotation around the single bonds. The IUPAC defines *s-cis*, *s-trans* conformation as: "the spatial arrangement of two conjugated double bonds about the intervening single bond is described as *s-cis* if syn-periplanar and *s-trans* if anti-periplanar" [35], see Figure 21.

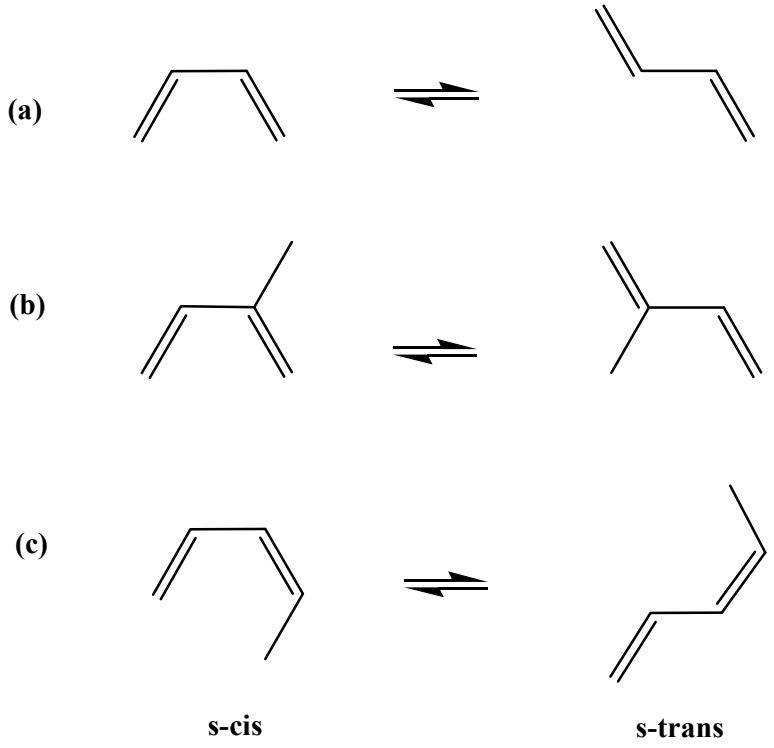


Figure 21. (a) 1,3-butadiene (b) 2-methyl-1,3-butadiene (c) 1,3-pentadiene.

The energetic barrier between the *s-trans* and *s-cis* conformations in simple conjugated systems like 1,3 butadiene is low. The enthalpy difference between the *s-trans* and *s-cis* was

calculated in an ab initio study using the basis set 3-61G** and it was estimated to be about 16.26 kJ/mol (3.89 kcal/mol) [36]. The classical model, for conjugated systems with methyl substitution, 2-methyl-1,3-butadiene is informative. The *s-trans* and *s-cis* conformers of 2-methyl-1,3-butadiene are separated by a barrier estimated to be about 24.2 kJ/mole (5.8 kcal/mol).

The addition of a methyl group in the system on C-2 has little effect on the enthalpy difference between the *s-cis* and *s-trans* conformers for these butadienes (Figure 21). The conformational equilibrium between the *s-cis* and *s-trans* conformers of 2-methyl-1,3-butadiene (see Figure 21b) has been calculated to be 1.78 ± 0.15 kcal/mol using an UV absorption spectroscopic method [37]. However, the ab initio study of the conjugated hydrocarbons, 2-methyl-1,3-butadiene and 1,3-pentadiene (methyl at C1 of 1,3-butadiene) showed that the energy difference of the *s-cis* and *s-trans* conformer are 3.38 kcal/mol and 3.87 kcal/mol [38]demonstrating the importance of the position of the methyl to the energy of these conformers. The presence of methyl substituents on the carotenoid polyene chain and the more extensive conjugation compared to these simple dienes is expected to significantly alter the energetics of the accessible conformations and the barrier to inter-conversion between conformers [39].

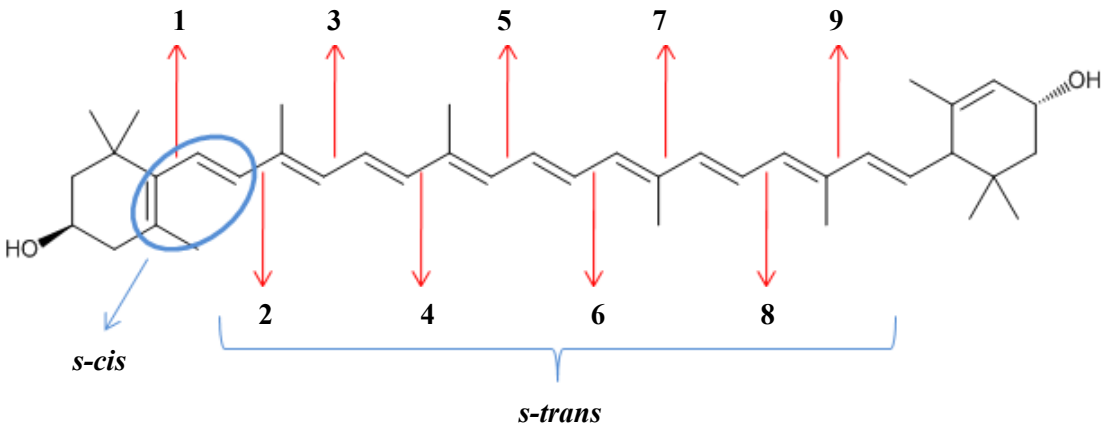


Figure 22. Lutein single bonds assigned numbers are shown by red arrows.

The existence of 9 ‘single’ bonds between the double bonds of the polyene chain (in lutein) makes possible the existence of numerous conformational geometries that correspond to rotation about these “single” bonds. In lutein, 512 conformations are possible, when considering the possible combinations of s-*cis* and s-*trans* conformations at the single bonds located along the backbone of the polyene chain.

In plants, fungi and invertebrates some carotenoid-binding proteins have been identified and characterized [40]. But in animals, carotenoid-binding proteins are still only poorly studied. Carotenoids in the circulation are transported in association with the lipoproteins. For example, the pathway that carotenoids like lutein and zeaxanthin follow to accumulate in the macula is not well-known. Serum lipoproteins and their receptors are known to be involved in the uptake and transportation of carotenoids in the humans and animals [41]. We can infer that the process of carotenoid binding to proteins requires dynamic mobility and their rapid interaction with the membrane proteins and enzymes within the photosynthetic apparatus of plants supports this conclusion. Since carotenoids can accumulate in cellular membranes, we also expect that their conformational flexibility will influence the rate and ability to repartition into other cellular organelles and proteins, both membrane-bound and water soluble. Thus, the affinity of carotenoids for binding to a protein and the rate of that process will be influenced by the energetic accessibility of conformations that favor the binding process. Carotenoids are often thought of as rigid, rod-like molecules, but they have a degree of flexibility that influences the dynamics and thermodynamics of their interaction with their environment. In this study, in an approach to develop a better understanding of how carotenoids interact with their environment and to highlight the importance of conformation in carotenoid binding, we conducted theoretical calculations on the energetics of conformations of the lutein. We calculated the minimum energy optimization profiles for all of the conformational geometries involving *s-cis* bonds. We created a

catalog including all the different combinations of *s-cis* conformers for lutein. In order to accomplish this we have conducted our calculation using the complete carotenoid structure (as opposed to small molecule fragments) with the semi-empirical parametric method 3 (PM3)[42]. To validate the semi-empirical approach we have also performed a number of calculations using density functional B3LYP/6-31G* level of theory. We used the program Gaussian 09 Revision A.02 [43] for the B3LYP/6-31G* calculations. Semi-empirical methods are most useful for a comparative or qualitative understanding and have limitations that can invalidate their application in certain cases or influence the applicability of the calculation to particular systems.

Semi empirical methods, such as PM3, use parameters derived from experimental data to simplify the calculations and approximations needed to solve the Schrodinger equation. The PM3 is a level of theory that can be readily used for large molecules. In the past, PM3 has been used to calculate optimized 3-dimensional geometries and energy state descriptors of molecules in Quantitative Structure-Activity Relationship (QSAR) studies [44]. PM3 calculations are based on mathematical approximations. Density functional theory, on the other hand, is widely accepted to have better accuracy than semi-empirical methods. They compute electron correlation using general functions of electron density [45]. Becke's three-parameter hybrid functional (B3LYP) is a hybrid functional and is used to determine the electron density within a molecule. [45]. The B3LYP method has been proven to be a superior approach when compared with the semi-empirical methods but is a time-consuming method. Density functional theory is more useful in that it can predict diverse molecular properties: vibrational frequencies, ionization energies and magnetic properties, etc. It has become more commonly used in computational chemistry over the past 10 years.

2.2. Research Methodology

2.2.1. Geometry optimization using the semi-empirical theory: PM3

Geometry optimizations were calculated for lutein conformers using the program Hyperchem 8 [http://www.hyper.com/?tabid=360] Lutein was modeled in Hyperchem and the initial geometry was optimized to the minimum energy using the molecular mechanics (MM^+) force field method. Once an optimized geometry was obtained by the MM^+ method, the energy of the molecule was minimized using the semi-empirical PM3 calculation.

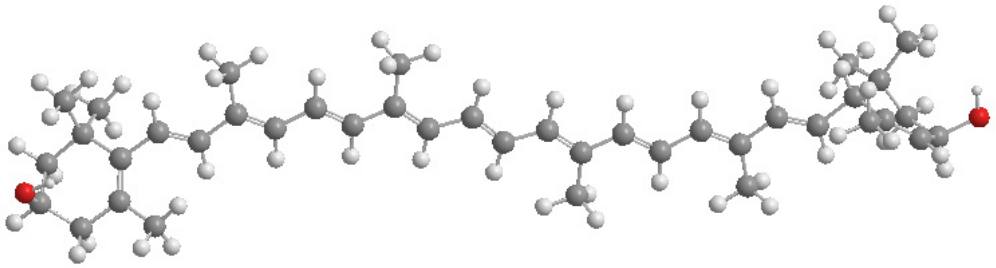


Figure 23. Optimized lutein model

After a geometry optimization of the all-*trans* structure was performed, we used that optimized lutein structure as the starting point to generate the various conformers by rotating around the single bonds in the molecule. The chosen bond(s) is set to 0° (the planar *s-trans* geometry is 180° and the s-*cis* is 0°). In the next step we allowed the calculation to find to the minimum energy of the geometry selected. For simplicity, in describing the various conformers, we number the nine single bonds within the polyene one through nine (see Figure 22) and name the conformers according to the single bond that has the *s-cis* conformation. For example, the conformer below is labeled tri-s-*cis*-2,4,9 because it is a molecule having three s-cis single bonds in the polyene system located at the 2^{nd}, 4^{th}, and 9^{th} single bonds as numbered from left (β-ring) to right (ε-ring) (Figure 24).

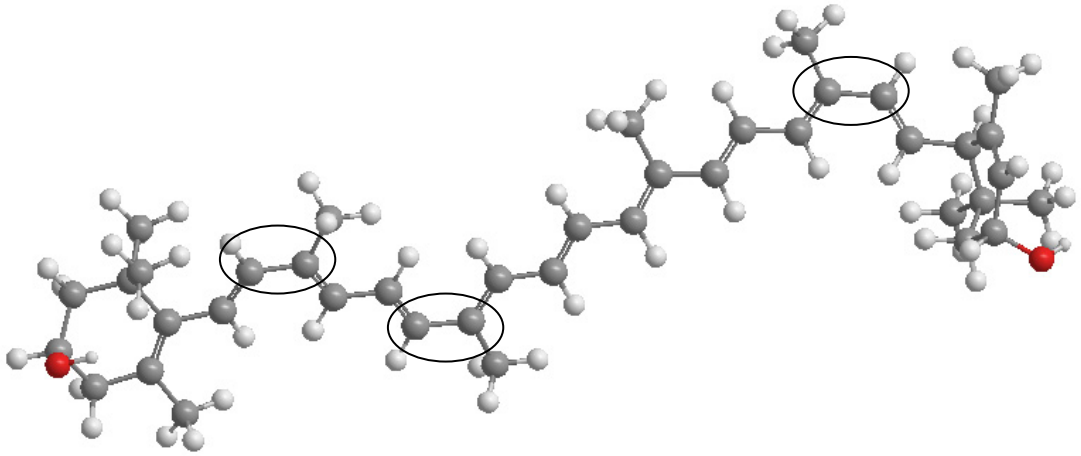


Figure 24: tri s-*cis* 2,4,9 lutein conformer.

The Tables numbered 4-27 are grouped according to the number of s-*cis* bonds present in the structures and provide a straight-forward way to group the data that resulted from our calculations. We have also created graphical representations that enable us to conceptualize a surface for each group of molecular conformers and recognize patterns that result from the placement of the s-*cis* bonds in different locations and/or combinations.

2.2.2. Geometry optimization using the density functional theory: B3LYP/6-31G*.

The results obtained from the PM3 calculations for some selected conformers were compared using the B3LYP/6-31G* computational method within the Gaussian 09 Revision A.02 program.

To run these calculations we used a Gaussian z-matrix format to specify the molecular geometry of the conformers. The z-matrix is derived by the Cartesian coordinates produced by the PM3 optimized geometries for the selected lutein conformers. The first line of the matrix, represented by two integers, specifies the charge and spin multiplicity of the molecule. The next lines indicate the atom, represented by its nominal atomic mass. And the location of the atoms is represented by the cartesian coordinates.

2.2.3. Calculation of the energy rotational barrier

The conformers that were selected for validation using B3LYP were chosen on the basis of their energy patterns. A number of conformers had remarkably low energies as calculated by the PM3 method and validation of these was an important step. Similarly, some of the conformers had surprisingly higher energies and these too needed to be validated. Energy barriers for rotation around specific single bonds were calculated using the B3LYP/6-31G* method at the following torsion angles: 0°, 30°, 60°, 90°, 120°, 150°, and 180°. A z-matrix was constructed for this purpose.

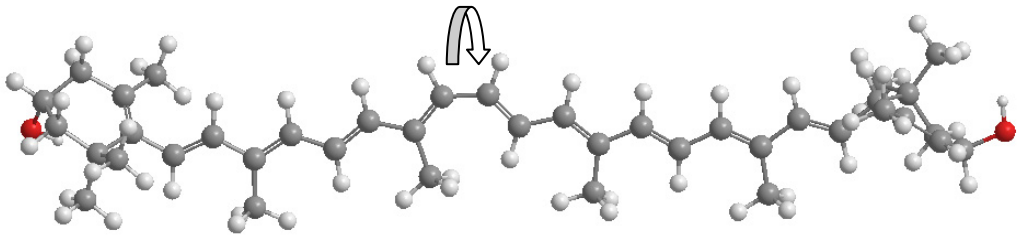


Figure 25: Rotation about the 5th single bond in lutein**.**

2.3. Results

2.3.1. Calculations for the basic conjugated system

Table 2 shows the calculations run for the s-*cis* and s-*trans* conformers of 1,3-butadiene, 2-methyl-1,3-butadiene, and 1,3-pentadiene (see Figure 21) using the PM3 method and Table 3 shows the calculations using B3*LYP theory.

Table 2: Calculations using PM3 theory.

PM3	1,3 butadiene	2-methyl-1,3-butadiene	1,3-pentadiene
s-cis **(kcal/mol)**	-964.5380	-1249.2777	-1248.4794
s-trans **(kcal/mol)**	-965.2665	-1248.7766	-1250.3684
ΔE kcal/mol	0.7285	0.5011	1.8990

Table 3: Calculations using B3*LYP theory.

B3*LYP	1,3 butadiene	2-methyl-1,3-butadiene	1,3-pentadiene
s-cis **(kcal/mol)**	-98038.7021	-122750.8496	-122749.6427
s-trans **(kcal/mol)**	-98042.5878	-122754.5361	-122754.1306
ΔE kcal/mol	3.8857	3.6865	4.4879

The relative energies are compared for each of the three cases. The absolute energies calculated by the two methods cannot be compared because we are using two different theories. The semi-empirical method only takes in account the valence electrons, while the density functional method uses all the electrons involved in the structure. This is why we can see a big difference between the values obtained in each case.

2.3.2. Calculations for the complete carotenoid model using PM3.

The following tables are grouped according to the number of s-*cis* bonds present in the structures. The tables indicate the *s-cis* location in the polyene chain (Figure 21) by the column and row respectively, and ΔE is given in kcal/mol. For example: if the first column is numbered 2, and the row 4 the conformer is di-*cis* 2,4 lutein. If the first column indicates 235 and the row 7 then the tetra-*cis*-2,3,5,7 lutein conformer is referred to. We have assigned the energy of the

predominant in nature tobe zero. That conformer is the molecule corresponding to mono-*cis* 1-lutein, notice that the *s-cis* bond is located at the beginning of the polyene chain (Figure 22, Figure 23).

Tables 4-27 show the results obtained using the PM3 calculation.

Conformers containing one *s-cis* single bond

Table 4: Relative energies for conformers containing one *s-cis* single bond.

***s-cis* location**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**all- *E* lutein**
ΔEnergy kcal/mol	0	-0.7644	2.7274	-0.6113	3.002	1.5183	0.1606	1.5491	0.3746	0.5387

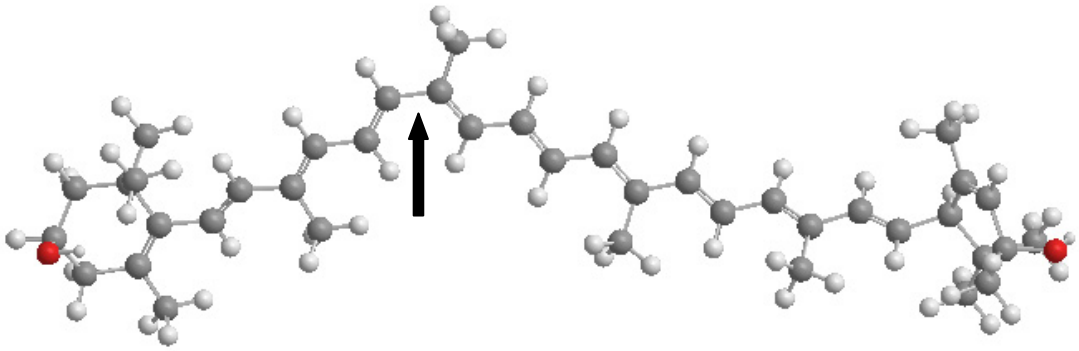


Figure 26: Example: mono s-*cis* 4 lutein conformer.

Conformers containing two *s-cis* single bonds

Table 5: Relative energies for conformers containing two *s-cis* single bonds.

s-cis location/ ΔE (kcal/mol)	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
1	0.2374	2.1976	-1.0314	2.4799	0.6376	0.2729	0.6117	0.4105
2		1.9600	-1.8991	1.9954	0.2798	-1.1254	-0.0537	-0.9086
3			1.6728	5.1335	3.6838	2.4816	3.8212	2.6000
4				2.2157	0.5759	-0.9789	0.5994	-0.8073

Table 5 continues:

	2	3	4	5	6	7	8	9
5					4.2252	-0.036	3.0676	1.315
6						1.1925	2.8143	1.3394
7							2.7073	-0.056
8								1.3619

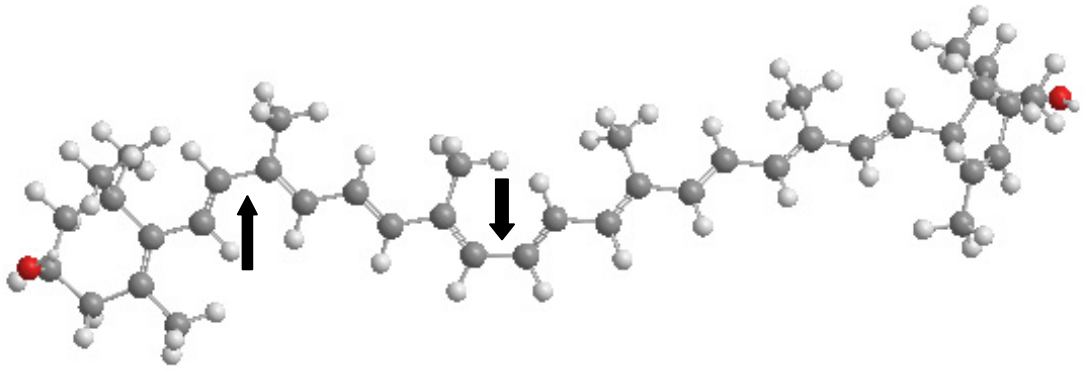


Figure 27: Example: di s-*cis* 2,5 lutein conformer.

Conformers containing three *s-cis* single bonds

Table 6: Relative energies for conformers containing three *s-cis* single bonds.

s-cis location/ ΔE (kcal/mol)	**3**	**4**	**5**	**6**	**7**	**8**	**9**
12	2.1239	-1.4354	2.4041	0.538	-0.8199	0.5504	-0.6838
13		1.5598	4.7342	2.8581	1.821	3.1677	1.9632
14			1.7444	0.1922	-1.3675	0.111	-1.2193
15				3.5971	2.0585	2.4551	2.2535
16					0.2948	2.0545	0.4807
17						1.5215	0.062
18							0.4827

Table 7: Relative energies for conformers containing three *s-cis* single bonds.

s-cis location/ ΔE(kcal/mol)	**4**	**5**	**6**	**7**	**8**	**9**
23	0.9026	4.4566	3.3257	1.7236	3.0586	1.8218
24		1.0048	-0.6424	-2.2371	-0.6756	-2.0602
25			2.8725	1.6004	2.8764	1.7667
26				-0.2303	1.4826	0.0477
27					0.1396	-1.3009
28						0.0362

Table 8: Relative energies for conformers containing three *s-cis* single bonds.

s-cis location/ ΔE(kcal/mol)	**5**	**6**	**7**	**8**	**9**
34	5.0609	2.5288	1.3426	2.6144	1.5149
35		6.2409	4.7733	6.1011	4.9292
36			3.0945	4.8855	3.5
37				4.0485	2.3182
38					3.5441

Table 9: Relative energies for conformers containing three *s-cis* single bonds.

s-cis location/ ΔE(kcal/mol)	**6**	**7**	**8**	**9**
45	2.455	1.757	2.9931	1.9936
46		0.0517	1.7433	0.3442
47			0.3476	-1.1515
48				0.2527

Table 10: Relative energies for conformers containing three *s-cis* single bonds.

s-cis location/ ΔE(kcal/mol)	**7**	**8**	**9**
56	3.8735	5.5269	4.0618
57		1.796	-0.2219
58			2.8145
67		2.55	1
68			2.5598
78			1.5593

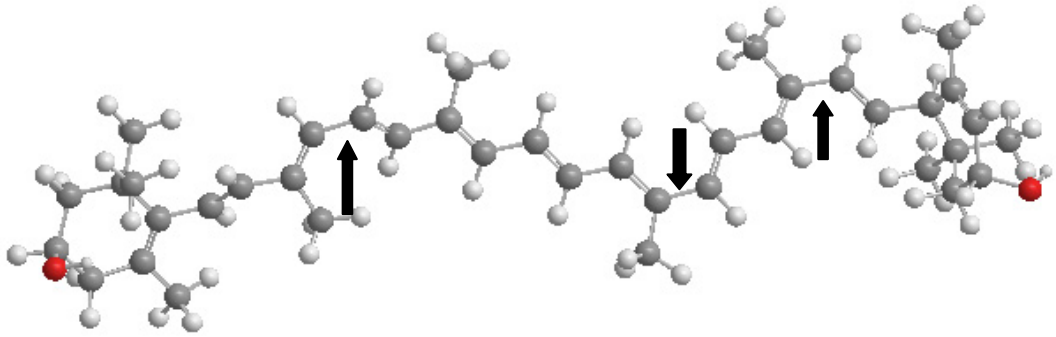


Figure 28: Example: tri s-*cis* 3,7,9 lutein conformer.

Conformers containing four *s-cis* single bonds

Table 11: Relative energies for conformers containing four *s-cis* single bonds.

s-cis location / ΔE (kcal/mol)	**4**	**5**	**6**	**7**	**8**	**9**
123	0.891	4.3172	2.7676	1.4953	2.8415	1.6552
124		0.8519	-0.7478	-2.2994	-0.8455	-2.1818
125			2.8691	0.2344	1.7645	1.6992
126				-0.1139	1.444	0.0031
127					-0.0171	-1.484
128						-0.0298
134		4.5207	2.0724	0.5046	1.7316	0.8168
135			5.3864	3.8934	4.6839	4.6046
136				2.5975	4.8767	3.3819
137					3.6693	1.7748
138						2.4645
145			2.6742	1.4906	2.752	1.5318
146				-0.074	1.4571	-0.0159
147					-0.0803	-1.5653
148						-0.0731
156				3.5572	4.1199	3.7065
157					4.1322	1.0315
158						2.4942
167					2.2851	0.6476
168						2.1906
178						0.804

Table 12: Relative energies for conformers containing four *s-cis* single bonds.

s-cis location/ ΔE(kcal/mol)	5	6	7	8	9
234	3.4489	1.6584	0.5832	1.9288	0.729
235		5.3743	4.1745	5.3789	4.2424
236			2.5971	4.4577	3.113
237				3.5382	1.5484
238					2.796
245		1.7451	0.5239	1.7278	0.7652
246			-1.2147	0.4745	-0.887
247				-0.9227	-2.4335
248					-0.9534
256			2.5609	4.1602	2.684
257				2.7431	1.405
258					2.6349
267				1.5175	-0.4189
268					1.2014
278					-0.0572

Table 13: Relative energies for conformers containing four *s-cis* single bonds.

s-cis location/ ΔE(kcal/mol)	6	7	8	9
345	4.894	4.5533	5.7078	4.8054
346		2.01	3.5202	2.2922
347			2.226	1.1479
348				2.3368
356		5.8798	7.3713	6.0545
357			5.85172	4.5883
358				5.8524
367			4.3631	2.8765
368				4.6341
378				3.5274

Table 14: Relative energies for conformers containing four *s-cis* single bonds.

s-cis location/ ΔE(kcal/mol)	7	8	9
456	2.1081	3.4651	2.2871
457		2.8032	1.5687
458			2.7528
467		1.6121	-0.1375

Table 14 continues:

	7	8	9
468			1.4659
478			0.1004
567		5.4027	3.6851
568			5.2868
578			1.4516
678			2.2355

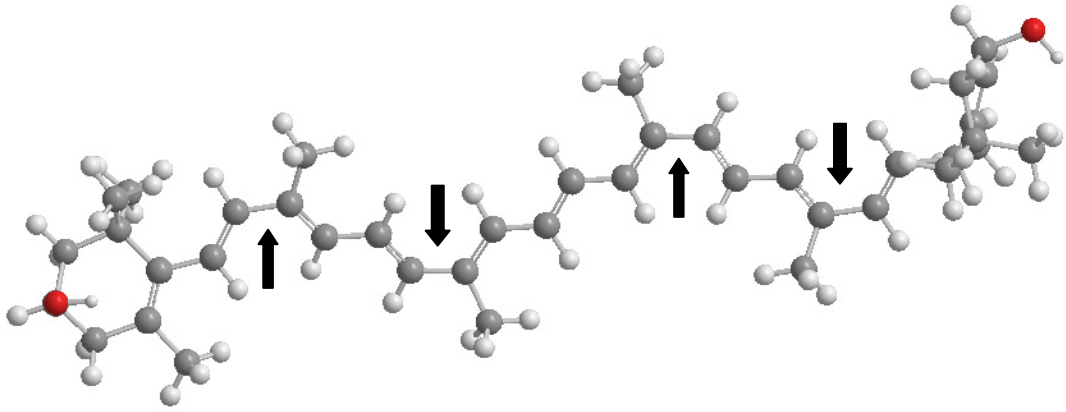


Figure 29: Example: tetra s-*cis* 2,4,7,9 lutein conformer.

Conformers containing five *s-cis* single bonds

Table 15: Relative energies for conformers containing five *s-cis* single bonds.

s-cis location/ ΔE(kcal/mol)	**5**	**6**	**7**	**8**	**9**
1234	4.1458	1.5726	0.4266	1.8952	0.6718
1235		5.3192	4.0035	5.2992	4.1063
1236			2.6439	4.3776	3.0791
1237				3.2642	1.2891
1238					2.6867
1245		1.726	0.5491	1.7905	0.6148
1246			-1.0246	0.5048	-0.8857
1247				-0.9936	-1.4938
1248					-1.0038
1256			2.5619	3.1239	2.7009

Table 15 continues:

	5	6	7	8	9
1257				3.4689	-0.0008
1258					1.5665
1267				1.2552	-0.3854
1268					1.2258
1278					-0.2397

Table 16: Relative energies for conformers containing five *s-cis* single bonds.

s-cis location/ ΔE(kcal/mol)	**6**	**7**	**8**	**9**
1345	4.745	4.2102	4.6313	4.402
1346		1.7095	4.8767	1.8766
1347			1.6804	0.5461
1348				1.5181
1356		5.6351	6.6292	5.1946
1357			6.2012	3.8939
1358				5.1679
1367			3.9311	3.8939
1368				4.5984
1378				3.0017
1456		2.3477	3.8456	2.4948
1457			3.4478	1.2961
1458				2.5098
1467			1.4401	-0.3649
1468				1.2254
1478				-0.2372
1567			4.8533	3.3456
1568				4.8819
1678				2.1387

Table 17: Relative energies for conformers containing five *s-cis* single bonds.

s-cis location/ ΔE(kcal/mol)	**6**	**7**	**8**	**9**
2345	4.2487	3.075	4.3159	3.2456
2346		1.2031	2.8189	0.4318
2347			1.5336	0.3886
2348				1.6271

Table 17 continues:

	6	7	8	9
2356		5.0534	6.6212	5.1865
2357			5.2466	3.9505
2358				5.1506
2367			3.5462	2.3872
2368				4.1668
2378				3.0161
2456		1.3459	2.968	1.5177
2457			1.5518	0.3425
2458				1.4685
2467			0.3582	-1.4008
2468				0.2191
2478				-1.2093
2567			5.2902	2.3739
2568				3.9208
2578				2.4988
2678				1.1785

Table 18: Relative energies for conformers containing five *s-cis* single bonds.

s-cis location/ ΔE(kcal/mol)	**7**	**8**	**9**
3456	4.5607	6.193	4.7207
3457		4.9047	4.3513
3458			5.4283
3467		3.8136	1.8258
3468			3.2204
3478			1.9921
3567		6.5296	5.6974
3568			6.5772
3578			5.611
3678			4.0288
4567		3.4767	1.943
4568			3.1782
4578			3.1782
4678			1.279
5678			5.0578

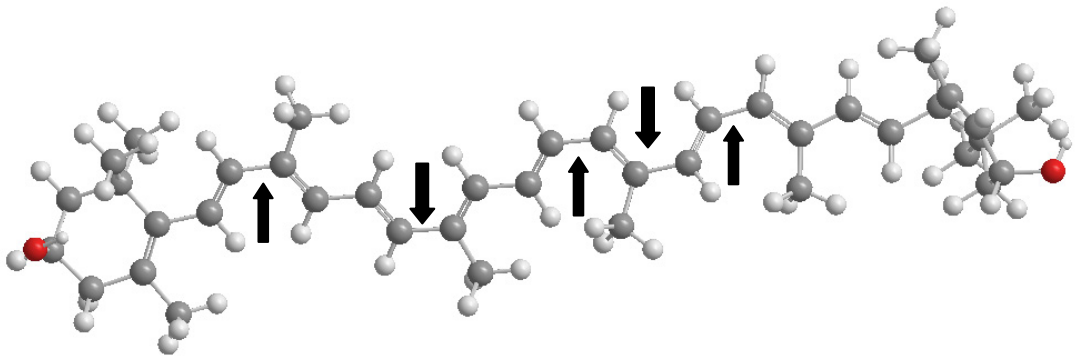


Figure 30: Example: penta s-*cis* 2,4,6,7,8 lutein conformer.

Conformers containing six *s-cis* single bonds

Table 19: Relative energies for conformers containing six *s-cis* single bonds.

s-cis location/ ΔE(kcal/mol)	**6**	**7**	**8**	**9**
12345	4.453	6.5185	4.8319	3.9057
12346		1.2583	2.8212	1.4489
12347			1.4377	0.2827
12348				1.6407
12356		4.9201	6.5185	5.1402
12357			3.861	3.8189
12358				5.2608
12367			3.4618	2.3996
12368				5.1402
12378				2.5247
12456		1.3826	2.9358	1.5565
12457			2.5375	0.3891
12458				1.598
12467			0.4022	-1.219
12468				0.2868
12478				-1.0997
12567			3.8902	2.3513
12568				3.8987
12578				3.203
12678				1.1188

Table 20: Relative energies for conformers containing six *s-cis* single bonds.

s-cis location/ ΔE(kcal/mol)	**7**	**8**	**9**
13456	4.4828	5.9184	4.4569
13457		4.2959	1.2269
13458			5.1771
13467		3.1152	1.6005
13468			3.2506
13478			1.5135
13567		6.2477	5.6817
13568			6.9354
13578			4.9041
13678			3.8372
14567		3.6349	2.1273
14568			3.6564
14578			3.3434
14678			1.4457
15678			4.9115

Table 21: Relative energies for conformers containing six *s-cis* single bonds.

s-cis location/ ΔE(kcal/mol)	**7**	**8**	**9**
23456	3.8312	4.9189	4.0351
23457		3.9544	2.887
23458			4.0788
23467		3.8851	1.0096
23468			2.5499
23478			1.3016
23567		6.556	4.8591
23568			6.3913
23578			5.0023
23678			3.2613
24567		3.0456	1.1578
24568			2.7271
24578			1.3186
24678			0.0425
25678			4.0707

Table 22: Relative energies for conformers containing six *s-cis* single bonds.

s-cis location/ ΔE(kcal/mol)	8	9
34567	7.1866	4.3861
34568		5.8982
34578		4.6781
34678		3.3729
35678		6.2676
45678		3.0356

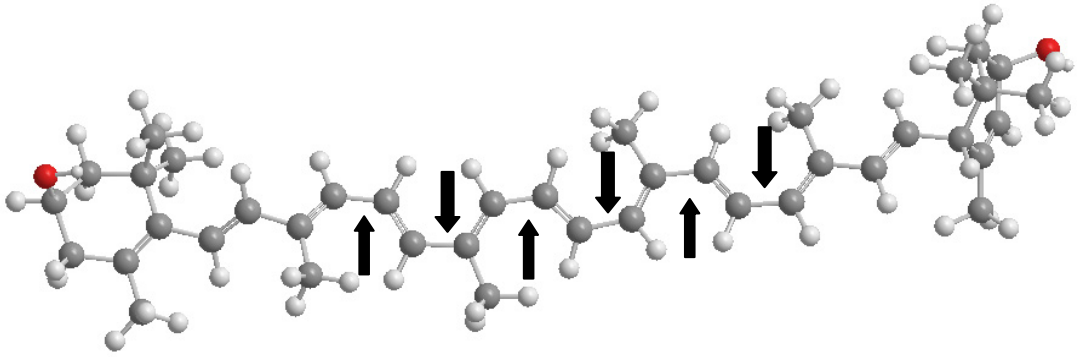


Figure 31: Example: hexa s-*cis* 3,4,5,6,7,8 lutein conformer.

Conformers containing seven *s-cis* single bonds

Table 23: Relative energies for conformers containing seven *s-cis* single bonds.

s-cis location/ ΔE(kcal/mol)	7	8	9
123456	4.1748	5.3929	4.0519
123457		5.2741	3.133
123458			4.5018
123467		2.6269	1.0052
123468			2.5759
123478			1.2664
123567		6.4002	4.7895
123568			6.3039
123578			5.5254
123678			3.2605
124567		2.6986	1.1635

Table 23 continues:

	7	8	9
124568			2.6661
124578			2.3972
124678			0.4631
125678			3.957
134567		5.4869	3.8966
134568			5.7017
134578			5.7188
134678			2.8482
135678			6.7334
145678			3.6977

Table 24 Relative energies for conformers containing seven *s-cis* single bonds.

s-cis location/ ΔE(kcal/mol)	**8**	**9**
234567	5.1413	3.6442
234568		4.6401
234578		3.735
234678		3.735
235678		6.2518
245678		2.6293
345678		5.9604

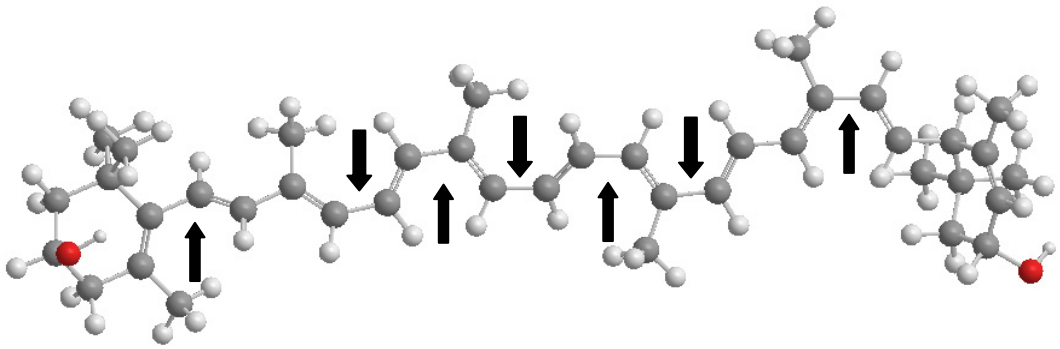


Figure 32: Example: hepta s-*cis* 1,3,4,5,6,7,9 lutein conformer.

Conformers containing eight *s-cis* single bonds

Table 25 Relative energies for conformers containing eight *s-cis* single bonds.

s-cis location	**12345678**	**12345679**	**12345689**	**12345789**	**12346789**
ΔEnergie (kcal/mol)	5.0392	3.7859	5.2398	5.3723	2.5761

Table 26 Relative energies for conformers containing eight *s-cis* single bonds.

s-cis location	**12356789**	**12456789**	**13456789**	**23456789**
ΔEnergie (kcal/mol)	6.3434	2.7517	5.6877	4.8792

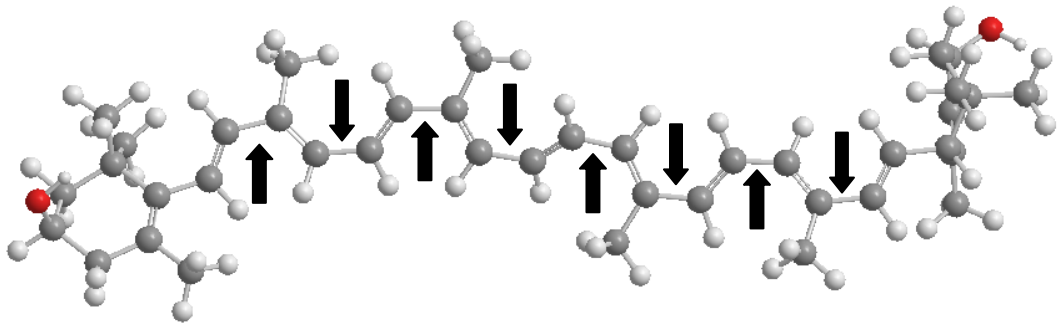


Figure 33: Example: octa s-*cis* 2,3,4,5,6,7,8,9 lutein conformer.

Conformer containing nine *s-cis* single bond

Table 27 Relative energies for the conformer with nine *s-cis* single bonds.

s-cis location	**123456789**
ΔEnergy (kcal/mol)	5.2324

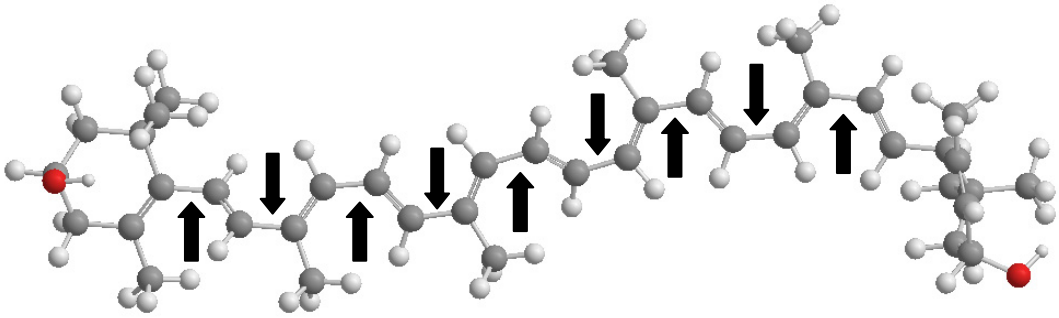


Figure 34: Example: nona s-*cis* 1,2,3,4,5,6,7,8,9 lutein conformer.

2.3.3. Calculations for the complete carotenoid model using B3*LYP/6-31G*.

From among all the conformers that the model can have we selected some of the conformers to compare the calculations run with the semi-empirical PM3 method and the B3*LYP method. The results are shown is Table 28.

Table 28: Calculations with B3*LYP for selected conformers of the lutein model**.**

Conformer	E (kcal/mol)	ΔE
lutein	-1073692.21	0.00
***all-E* lutein**	-1073689.81	2.39
2	-1073687.09	5.12
3	-1073685.34	6.87
4	-1073687.43	4.78
5	-1073685.93	6.28
6	-1073685.89	6.32
7	-1073687.26	4.95
8	-1073685.48	6.73
9	-1073687.25	5.15
24	-1073684.05	8.16
26	-1073682.80	9.41
27	-1073684.04	8.15
35	-1073681.17	11.04
49	-1073683.60	7.92

56	-1073680.69	11.51
68	-1073681.32	10.89
247	-1073680.67	11.54
248	-1073679.40	12.81
249	-1073680.88	11.33
368	-1073676.47	15.74
379	-1073679.44	12.77
479	-1073679.99	11.35
489	-1073680.03	12.18
1269	-1073681.47	10.74
2479	-1073677.43	14.78
3568	-1073670.95	21.26
4678	-1073675.00	17.20
13489	-1073677.36	14.84
23456	-1073670.97	21.24
24678	-1073671.53	20.68
234568	-1073665.08	27.12
345678	-1073666.39	25.82
356789	-1073665.14	27.07
456789	-1073669.12	23.08
2356789	-1073662.61	29.60
123456789	-1708.26709	29.60

2.3.4. Calculations for the rotational barrier of the single bonds in lutein model using the B3*LYP method

Calculations to find the rotational barrier for the 4th (C12-C13) and 5th (C14-C15) bond were run using the B3*LYP method. Table 29 shows the results for each case and Figure 35 shows the torsional potential for the bonds.

Table 29: Calculations for dihedral angle for the 4th and 5th single bond in the lutein model**.**

	4th single bond C12-C13		5th single bond C14-C15	
dihedral angle	**E (kcal/mol)**	**ΔE**	**E (kcal/mol)**	**ΔE**
lutein *trans* (180)	-1073690.89	0	-1073690.90	0
150	-1073689.51	1.38	-1073689.88	1.02
120	-1073683.90	6.99	-1073684.41	6.49
90	-1073681.55	9.35	-1073681.16	9.74
60	-1073685.34	5.55	-1073684.07	6.83
30	-1073688.42	2.47	-1073687.04	3.86
0	-1073688.70	2.19	-1073687.20	3.70

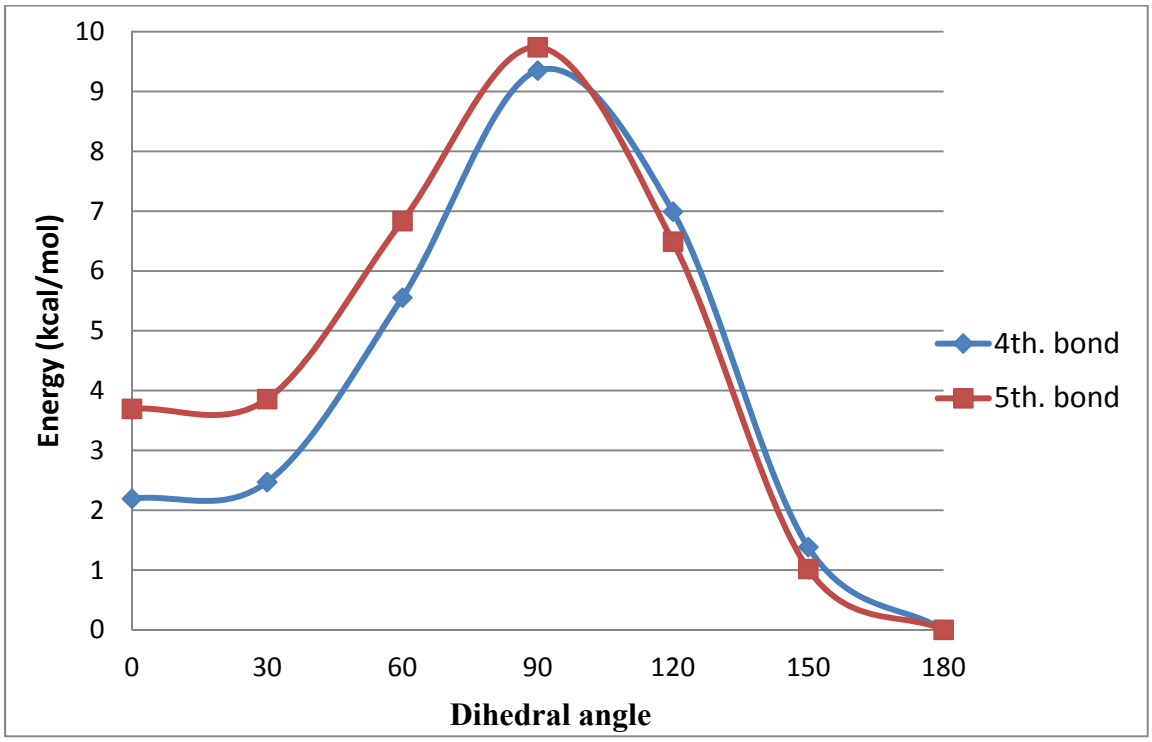


Figure 35: Energy barrier for the rotation of the single bond at the 4th and 5th bond in the lutein model.

2.4. Discussion

Below, Figure 36 maps the energy of the lutein conformers according to the numbers of *s-cis* bonds present. From the figure, we can see that the distribution of the energies of the lutein conformers lay mostly between 0 and 4 kcal/mol.

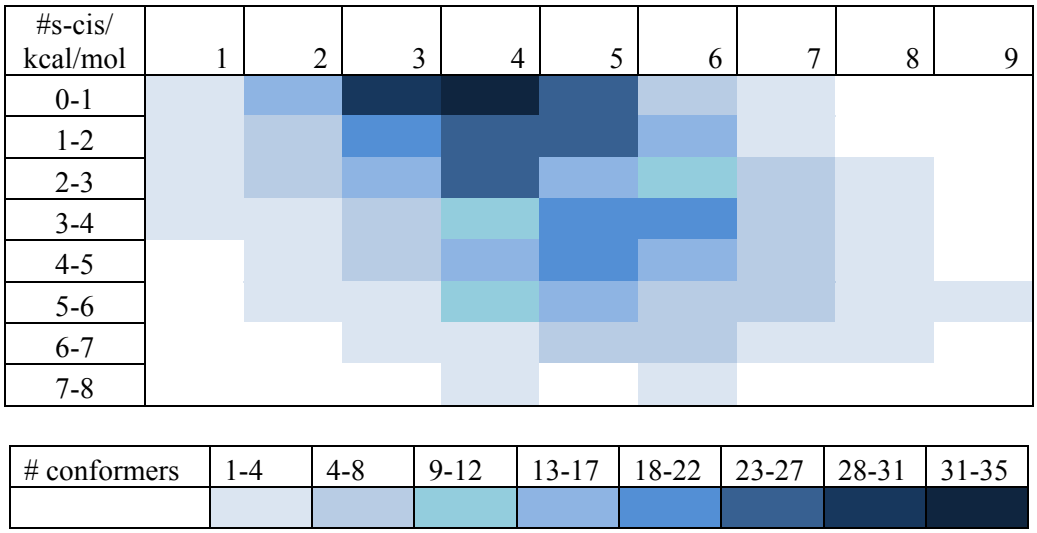


Figure 36: The energy of the lutein conformers according to the number of *s-cis* bonds and their location within the molecule.

A surprising observation is that the conformer containing 9 *s-cis* bonds is lower in energy than some others having 3 or 6 *s-cis* bonds in their structures. The conformer with the highest energy according to the PM3 method is the tetra *s-cis* 3568 lutein conformer and not the nona *s-cis* 123456789 lutein conformer, as we might have predicted. Another result observed about the conformers is that the position and arrangement of the methyl groups affects the stability of the different conformers. In lutein we can separate s-single bonds into two groups. In the first group, methyl substitution at single bonds 2, 4, 7, and 9 reduces the energy differences between the *s-cis* and *s-trans* conformers. In contrast the second group of single bonds, those labeled 3, 5, 6, and 8 lack a methyl resulting in a more dramatic difference in the energies of the two conformers

because the alkyl chains are oriented away from each other in the *s-trans* conformation. Bonds 2, 4, 7, and 9 are analogs to 2-methyl-1,3-butadiene (see Figure 21.b) and 3, 5, 6, and 8 (see Figure 21.c) are analogs to 1,3-pentadiene. If we take 1,3-butadiene as a reference we can say that the substitution at the second carbon lowers the energy for the isomerism *cis-trans* compared to substitution of a methyl group at the first carbon (1,3 pentadiene). The energies for the *cis-trans* conformers of the substituted 1,3-butadiene (Table 3) show that there is an approximate 1 kcal/mol difference between them. Conformers with the s-*cis* bonds in the first group of single bonds have lower energy.

Table 30: Correlation of ΔE (kcal/ mol) values between *s-cis* and *s-trans* conformers as calculated using PM3 and B3LYP/6-31G*.

	conformer	PM3	B3LYP/6-31G*	
minimum	**Lutein**	0.00	0.00	
One *s-cis* bond	**lutein trans**	0.54	2.39	
	2	-0.76	5.12	**Group I**
	4	-0.61	4.78	
	7	0.16	4.95	
	9	0.37	5.15	
	3	2.73	6.87	**Group II**
	5	3.00	6.28	
	6	1.52	6.32	
	8	1.55	6.73	
Two *s-cis* bond	**24**	-1.90	8.16	**Group I**
	27	-1.13	8.15	
	49	-0.81	7.92	
	26	0.28	9.41	
	35	5.12	11.04	**Group II**
	56	4.23	11.51	
	68	2.81	10.89	

Three *s-cis* bonds	**247**	-2.24	11.54	**Group I**
	249	-2.06	11.33	
	479	-1.15	11.35	
	248	-0.68	12.81	
	379	2.32	12.77	
	489	0.25	12.18	
	368	4.89	15.74	**Group II**
Four *s-cis* bonds	**1269**	0.00	10.74	
	2479	-2.43	14.78	
	3568	7.37	21.26	
	4678	1.61	17.20	
Five *s-cis* bonds	**13489**	1.52	14.84	
	23456	4.25	21.24	
	24678	0.36	20.68	
Six *s-cis* bonds	**234568**	4.92	27.12	
	345678	7.19	25.82	
	356789	6.27	27.07	
	456789	3.04	23.08	
Seven *s-cis* bond	**2356789**	6.25	29.60	
All *s-cis*	**123456789**	5.23	29.60	

Table 30 compares the ΔE values obtained using PM3 and B3LYP/6-31G* theories. When we compare the values using both methods, we notice that the values for and B3LYP are larger. But the tendency when we subdivide them is similar. In the first group of conformers, those with one *s-cis* bond (Table 30 have a lower relative energy than those in group II. We can see that this tendency repeats for all of the conformers in Table 30. The relative energy of the conformers that possess s-*cis* bonds that correspond to both group I and group II are intermediate in value compared to the lowest and the highest relative energies. Using the values from Table 30, we observed a good correlation allows use of the PM3 values to predict the B3LYP/6-31G* relative energy for each lutein conformer. The PM3 relative energy corresponds to the variable m and the number of s-cis bonds is represented by n in the conformer in the equation below:

B3LYP Energy = 4.14n + m

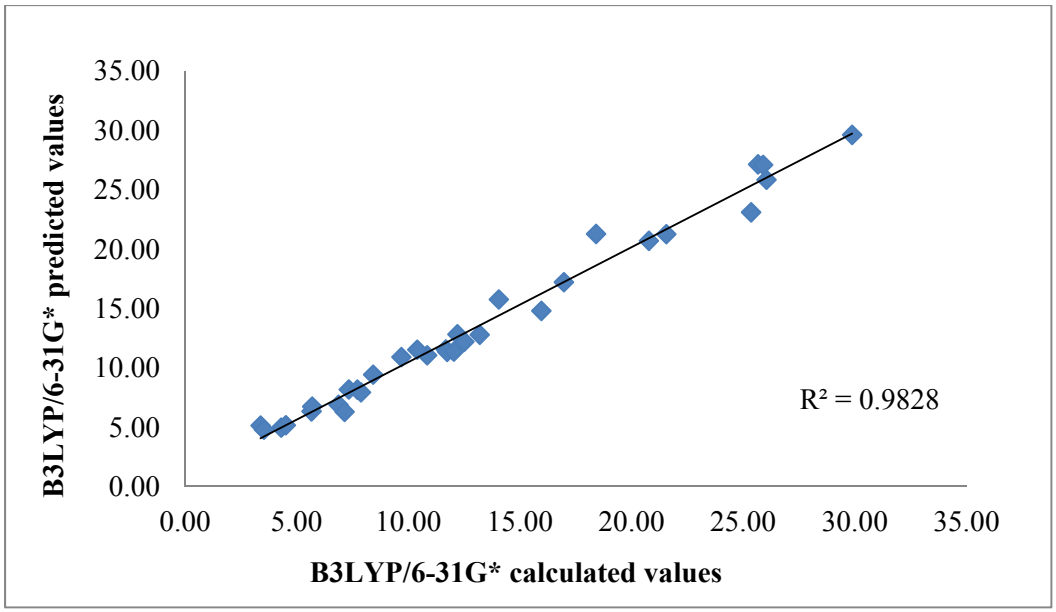


Figure 37: Correlation between the calculated B3LYP/6-31G* DE values and the predicted values using the formula.

Figure 37 shows the correlation of the predicted values based on PM3 and the actual B3LYP results. The values for PM3 are not as precise as the values that are obtained using the density functional theory. When using the B3LYP/6-31G* theory, the values of energy in kcal/mol are higher than those obtained by the PM3 method. The square of the correlation coefficient (R^2 = 0.9828) in the graph demonstrates that quality of the formula in estimating the computed B3LYP value. The range of relative energies calculated is from 2 to 30 kcal/mol for the different conformers using the B3LYP/6-31G* theory. The most accessible conformers are those that fall below 10 kcal/mol. We know that other molecules in nature readily overcome barriers of this magnitude and equilibration of conformations is rapid.. Cyclohexane is an example. The highest energy cyclohexane conformers, the twist chair, are 10.7 kcal/mol higher than the more preferred chair geometry. In the case of the lutein conformers, the mono and di-*cis*, conformers are below 10 kcal/mol in energy. Therefore, these conformers can co- exist although the higher

energy s-*cis* will be present in relatively small percentages by comparison to the preferred lutein conformer. The conformers with three s-*cis* bonds are in a range of 10 to 15 kcal/mol. The rest of the conformers have higher values. Although, it is interesting to note that the tetra s-*cis* 1269 has energy of only 10.74 kcal/mol. When we focus our attention on those conformers that have the s-*cis* single bonds in the group one (i.e. methyls at positions: 2, 4, 7, and 9), as we mentioned before, we can see that the tendency is to have a lower energies in comparison with those in the second group (i.e. methyls at positions: 3, 5, 6, and 8). This explains why the tetra s-*cis* 1269 lutein conformer has such a low energy relative to the most stable lutein conformer.

Figure 35 shows the rotational barriers that must be overcome for the s-*cis* single bonds to undergo interconversion to different conformations. The s-*cis* bonds analyzed are present at 4th and 5th position in the lutein model. As we mentioned before, the first one, shown in blue (4th position) is the analogue of 2-methyl 1,3-butadiene. Calculations made for the rotational barrier of this molecule [46] using *ab initio* quantum method show a maximum for the gauche conformation of 5.6 kcal/mol in the vicinity of 90-100°; and the minimum of 2.3 kcal/mol around 45° from the *syn* conformation (*s-cis*). The second one at the 5th position is shown in red and the rotational barrier for its analogue, 1,3 pentadiene was calculated using a *ab initio* theory at a MP2/6-31G* level [38]. The maximum energy is 5.9 kcal/mol for the gauche conformation at 100° and the minimum energy is 2.6 kcal/mol at 40°. For both simple models the curves are similar, as is the case for the two *s-cis* single bonds analyzed in this study. Although, the energy values of the carotenoids are higher when compared to the simple small molecule models, both of the curves have a similar pattern. The maximum energy in both cases was determined to be at 90° and the difference between them is about 0.39 kcal/mol. The higher relative energies can be attributed to the steric repulsions between the methyl groups in the long lutein model.

According to the results from the study conducted here, we propose that some of the *s-cis* conformers, that are sufficiently low in energy, exist in nature. Lutein participates in cellular activities and interacts with proteins and enzymes in the living systems. As a result of these types of interactions at a cellular level, lutein may adapt and adjust to that biological environment by dynamically deforming between a number of different low energy conformers. The significance of this work is that we may expect carotenoids to be stabilized in different conformations by interaction with biological molecules. These different conformations can be expected contribute to the physical and chemical properties as well as the behavior of the carotenoids within the biological environment. Binding of the carotenoid to a protein might readily modulate such important properties as excited state energies by favoring an s-*cis* conformer in preference to the native s-*trans* conformations.

CHAPTER 3: MEASUREMENT OF CAROTENOID LEVELS IN HUMAN SERUM

3.1. Overview:

Special attention has been paid to carotenoids including: lycopene, α- and β- carotenes for their response to minimize the risk of cancer and cardiovascular diseases. For the past two decades, many study trials [28, 47-49] in lutein supplementation among healthy subjects and patients with age-related macular disease in early and advanced stages have been carried out to investigate the response of lutein supplementation. Amounts of free lutein given to the subjects vary from 10 – 30 mg/day and the studies vary in short-term (3 months) to up to 4 years long-term periods [30, 50]. In all the cases, the relationship between the macular pigment caotenoids and AMD has been consistent and therefore, it has been suggested that the risk of Age-related macular degeneration can be reduced by increasing the intake of lutein. Increases in the amount of macular pigment located in the human retina protect these tissues from damage by photoxidation. In addition, the effect of multinutrients (including carotenoids) has been studied in HIV patients [51, 52]. Consequently, lutein supplementation studies are important to the nutritional field. The study of carotenoid levels in human serum is well documented and different methods have been published [53-55]. The dietary intake of lutein in the Western human diet is low [56, 57].

In this chapter, we studied six major carotenoids in human serum of subjects that participated in two lutein supplementation studies. We measured the carotenoid levels of lutein (L), zeaxanthin (Z), lycopene (lyc), β-carotene (β-C), α-carotene (α-C), β-cryptoxanthin (β-cry), and α-cryptoxanthin (α-cry) and determined the ratios both before and during supplementation. In addition, the macular pigment optical density (MPOD) of the subjects was measured by heterochromatic flicker photometry. The results are compared to determine the effect of the supplementation on elevating the MPOD of the subjects.

Table 31: Uv-vis data for carotenoids dissolved in ethanol [58] .

Carotenoid	λ_{max} (nm)
Lutein	422 445 474
Zeaxanthin	428 450 478
α – cryptoxanthin	423 446 473
β – cryptoxanthin	428 450 478
Lycopene	446 472 503
α – carotene	423 444 473
β – carotene	450 476

3.2. Research Methodology

3.2.1. Chemicals and instruments:

Lutein, dionized water, ethyl alcohol (USP grade), 1-pentanol 99%, hydrochloric acid, and triethylamine (TEA). Hexane, dichloromethane, methanol, acetronitrile, all HPLC grade. High pressure liquid chromatography (HPLC) system equipment used for carotenoid analysis: Waters 2690 couple to a photodiode array detector (PDA) Waters 996. High pressure liquid chromatography (HPLC) system used for standard purification: LDC/Milton Roy CM 4000 multiple solvent delivery system, Thermo Finnigan Spectra System UV 1000 detector. Ultraviolet-visible (UV-vis) spectrophotometer Cary 100 Bio.

3.2.2. Subjects:

Study I: 32 subjects (15 males and 17 females) were recruited from the FIU community for the study. Subjects were randomly assigned in one of the three groups: Group P1 was given placebo pills, and consisted of 10 subjects (4 males and 6 females). Group L1 was supplemented with pills containing lutein (20 mg/day), and consisted of 11 subjects (5 males and 6 females).

Group L2 was supplemented with diacetate lutein pills (20 mg/day), and consisted of 11 subjects (6 males and 5 females). Blood samples were collected before the supplementation and every 6 weeks after supplementation for 24 weeks in total. Macular pigment optical density was measured by heterochromatic flicker photometry before supplementation and every 6 weeks after supplementation for each subject.

Study II: 29 subjects (22 males and 7 females) were recruited from the FIU community, as well for this study. Subjects were randomly assigned in one of the three groups: Group P2 was given placebo pills, and consisted of 9 subjects (6 males and 3 females). Group L3 was supplemented with pills containing lutein diester (9 mg/day), and consisted of 10 subjects (8 males and 2 females). Group L4 was supplemented with pills containing lutein diester (9 mg/day), and additional, low level nutritional components, including lactoferrin, cassis extract, eyebright extract, royal jelly, coenzyme Q10. Study II consisted of 10 subjects (8 males and 2 females). Blood samples were collected before the supplementation and every 6 weeks after supplementation for 24 weeks in total. Macular pigment optical density was measure by heterochromatic flicker photometry before supplementation and every 6 weeks after supplementation for each subject.

3.2.3. Internal standard preparation:

Monopentyl lutein ether (MPL) was used as an internal standard for this study. In a flask, we dissolved 5 mg of lutein in 20 mL of 1-pentanol, and 1% v/v of hydrochloric acid was added. The reaction was placed for 3 hours in the dark and under stirring. Then, the solution was extracted three times with a solution of dichloromethane and deionized water (1:1). The product was concentrated and redissolved with ethanol to be purified by reversed phase HPLC. The concentration was determined by UV-visible spectrophotometry.

3.2.4. Serum extraction:

Blood samples were collected from the subjects and the serum was separated by centrifugation. The serum samples were collected in 2mL polypropylene vials, labeled, and stored at -80°C in the freezer. The carotenoids were extracted according to previous published technique [53]. The sample was thawed at room temperature and vortexed. An aliquot of 200µL of serum was placed in a glass tube, 35 µL of MPL and 2 mL of a mixture of ethanol/dionized water (1:1) was added to the tube. The tube was vortexed for 30 seconds and carotenoids were extracted three times with 2 mL of hexane. For every extraction the tube was vortexed and centrifuged for 5 minutes. The top layer of the separation was removed every time and collected in a 2mL vial. The aliquot obtained was dried under nitrogen gas flow. The sample was redissolved in 50 µL of ethanol and transfered to a vial for HPLC injection.

3.2.5. Carotenoid analysis:

The samples were analyzed by reversed phase HPLC, using a Phenomenex ODS ultracarb 3 µm column (20) of 250 x 4.60mm. The mobile phase was a mixture of acetonitrile/methanol/TEA (85% : 15% : 0.1% v/v). The flow rate was 1 mL/minute and the sample was run at 30°C for 70 minutes. The results were obtained from the chromatogram and peaks were labeled consistent with their retention time.

3.2.6. MPOD measurements [59, 60]:

The heterochromatic flicker photometry (HFP) is the method used to determine the MPOD. It can determine the spectral sensitivity of a human eye. In heterochromatic flicker photometry, lights of different spectral composition are alternated, and the subject has to adjust their relative radiances until the sensation of flicker is minimized or abolished. The two spectral mixtures are then defined as of equal luminance for that observer. To define a spectral sensitivity

function, different monochromatic lights (460 and 540 nm, in this case) are alternated with some standard. The flicker frequency is also an important parameter for the response to be reliable. The response defines the human photopic luminosity function that represents the measure of the average human visual sensitivity. The function is measure in two visual fields: small (2°) and large (10°). When it is measured using the small visual field it has an effect on the macular pigment but is quite insignificant in the large field. Therefore the difference of both functions gives a measure of the MPOD.

The subject's eye was covered with an eye patch and the subject was allowed to fix the focus of the stimulus with a hand-controlled button. Looking at the center of the stimulus the subject adjusts the setting until a flicker null is perceived and the wedge setting is recorded. This method is repeated five times in each: the small and large visual field. The MPOD of each subject were measured before and six weeks each time after supplementation for a total of 24 weeks period.

3.3. Results:

3.3.1. Characterization of the standard

The UV-Visible spectrum shown below corresponds to the standard prepared.

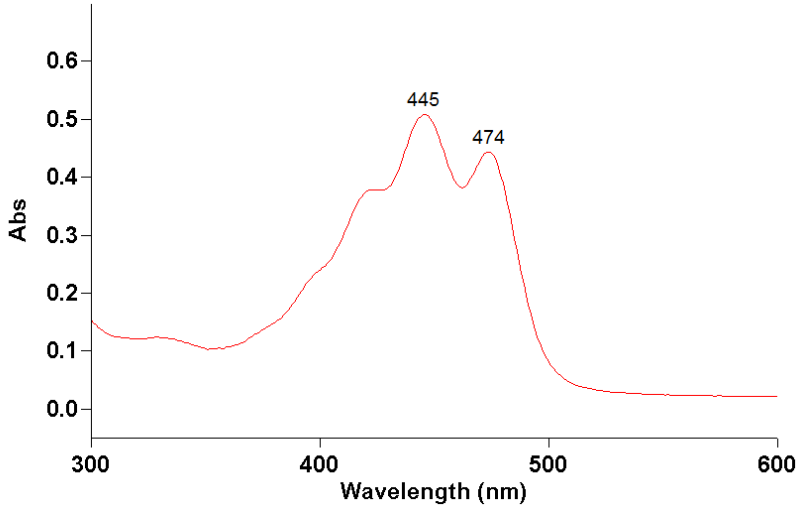


Figure 38: Monopentyl lutein UV-visible spectrum.

The spectrum is the same as that of lutein. The concentration of the standard was determinate using the Beer-Lambert law with the absorbance response at 450nm.

3.3.2. Baseline

The chromatogram below is a characteristic serum response of one of the subjects in the studies. The peaks were assigned according to their UV-visible spectra.

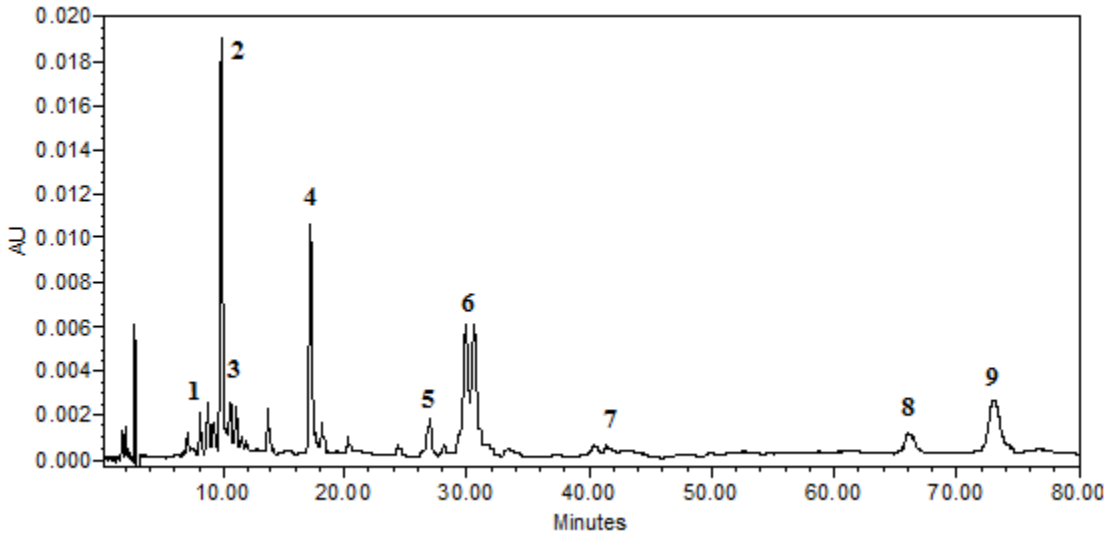


Figure 39: HPLC chromatogram of serum from subject before supplementation.
1) lutein metabolites. 2) lutein. 3) zeaxanthin. 4) α – cryptoxanthin. 5) β – cryptoxanthin. 6) MPL. 7)lycopene. 8) α – carotene. 9) β – carotene.

The picture below shows the expanded chromatogram from 0 – 12 minutes. The macula pigment carotenoids peaks are shown.

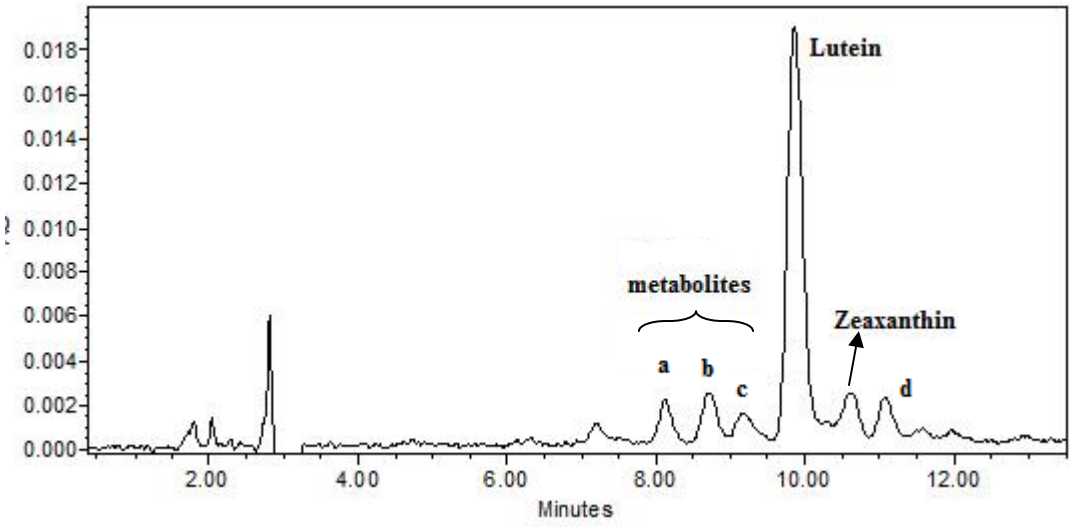


Figure 40: Expanded HPLC chromatogram of serum from subject before supplementation a) unknown. b) oxolutein. c) unknown d) 9Z lutein.

Table 32: Retention time of carotenoids in human serum.

Carotenoid	Retention time (min)
Lutein metabolites	8.00-9.00
Lutein	9.85
Zeaxanthin	10.61
α – cryptoxanthin	17.13
β – cryptoxanthin	26.88
Monopentyl lutein ether (MPL)	30.61
Lycopene	40.35
α – carotene	66.21
β – carotene	73.08

The distribution of the carotenoids in serum at baseline for all the groups that participate in both studies is shown below (for numeric values, refer to table 34).

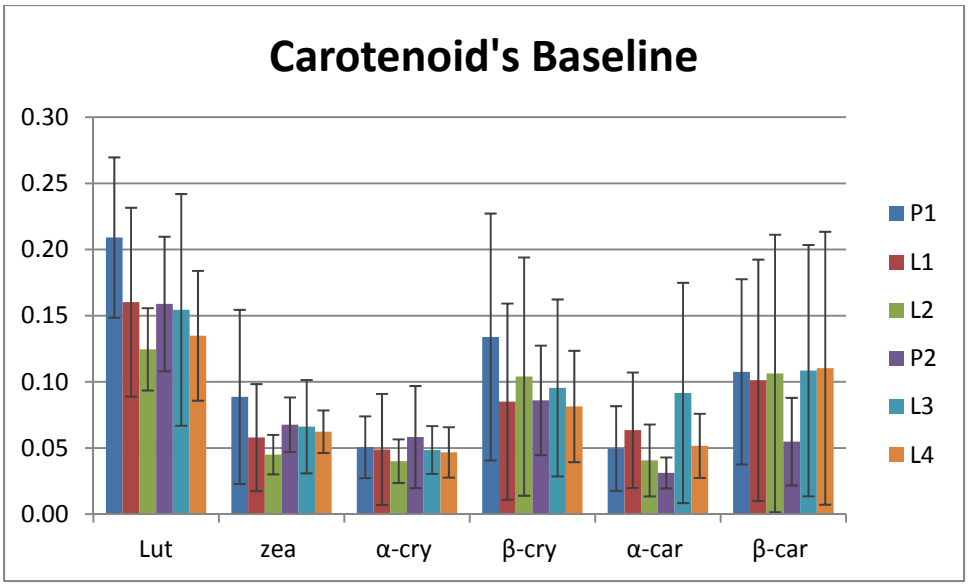


Figure 41: Distribution of the carotenoids at baseline according to the group. P1,placebo study 1. P2, placebo study II. L1 and L2, correspond to study I. L3 and L4, correspond to study II.

The ratios for lutein/zeaxanthin at baseline for all the studied groups vary between 2.2 to 2.8.

Table 33: Lutein/ zeaxanthin ratios for each study group.

	P1	L1	L2	P2	L3	L4
L/Z	2.36	2.77	2.77	2.35	2.33	2.16

Table 34: Characteristic serum concentrations of carotenoids at baseline.

	Study I			**Study II**		
Carotenoid concentration/ study group	P1	L1	L2	P2	L3	L4
Lutein (ng/µL)	0.21± 0.06	0.16 ± 0.07	0.12 ± 0.03	0.16 ± 0.05	0.15 ± 0.09	0.13 ± 0.05
Zeaxanthin (ng/µL)	0.09 ± 0.07	0.06 ± 0.04	0.05 ± 0.01	0.07 ± 0.02	0.07 ± 0.04	0.06 ± 0.02
α-crytoxanthin (ng/µL)	0.05 ± 0.02	0.05 ± 0.04	0.04 ± 0.02	0.06 ± 0.04	0.05 ± 0.02	0.05 ± 0.02
β-cryptoxanthin (ng/µL)	0.13 ± 0.09	0.09 ± 0.07	0.10 ± 0.09	0.09 ± 0.04	0.10 ± 0.07	0.08 ± 0.04
α-carotene (ng/µL)	0.05 ± 0.03	0.06 ± 0.05	0.04 ± 0.03	0.03 ± 0.01	0.09 ± 0.08	0.05 ± 0.02
β-carotene (ng/µL)	0.11 ± 0.07	0.10 ± 0.10	0.11 ± 0.10	0.05 ± 0.03	0.11 ± 0.09	0.11 ± 0.10

No statistical difference was found between any of the groups at the baseline level when analyzed the data by one-way ANOVA ($p > 0.60$).

3.3.3. Supplementation Response

The supplementation responses were recorded every 6 weeks for a period of 24 weeks. For a better understanding the data is presenting by groups.

Study I:

P1 group (placebo)

Table 35: Carotenoid concentrations in P1 throughout the study period (mean ± standard deviation).

Week	Lut (ng/µL)	zea (ng/µL)	α-cry (ng/µL)	β-cry (ng/µL)	α-car (ng/µL)	β-car (ng/µL)
6	0.19 ± 0.06	0.07 ± 0.03	0.05 ± 0.03	0.15 ± 0.11	0.03 ± 0.02	0.09 ± 0.08
12	0.23 ± 0.11	0.07 ± 0.04	0.07 ± 0.05	0.13 ± 0.09	0.04 ± 0.02	0.05 ± 0.06
18	0.20 ± 0.11	0.06 ± 0.03	0.08 ± 0.06	0.09 ± 0.07	0.03 ± 0.02	0.04 ± 0.04
24	0.15 ± 0.06	0.06 ± 0.02	0.06 ± 0.04	0.09 ± 0.09	0.06 ± 0.07	0.06 ± 0.06

No statistical difference ($p > 0.09$) was found between the baseline and the response after supplementation for all the carotenoids in this group.

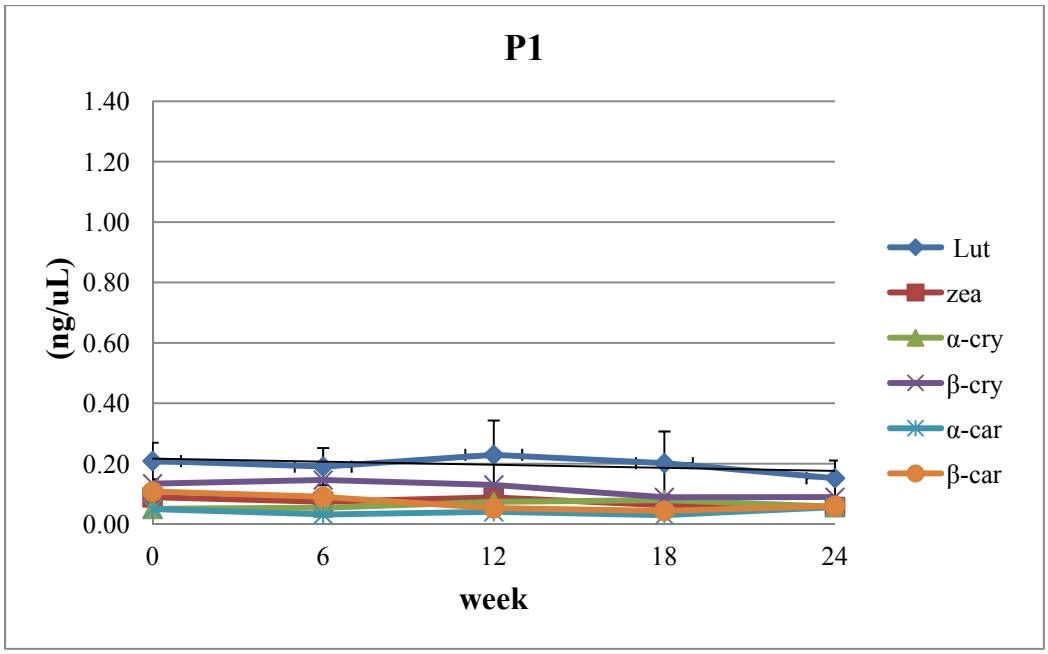


Figure 42: Comparison of carotenoids concentration in placebo group P1. Numeric values are shown in table 4.

L1 group

Table 36: Carotenoid concentrations after supplementation with L1 (mean ± standard deviation).

Week	Lut (ng/µL)	zea (ng/µL)	α-cry (ng/µL)	β-cry (ng/µL)	α-car (ng/µL)	β-car (ng/µL)
6	0.67 ± 0.35	0.09 ± 0.05	0.05 ± 0.02	0.09 ± 0.09	0.03 ± 0.01	0.08 ± 0.04
12	0.72 ± 0.37	0.14 ± 0.06	0.06 ± 0.03	0.09 ± 0.07	0.08 ± 0.08	0.03 ± 0.01
18	0.68 ± 0.46	0.14 ± 0.11	0.09 ± 0.05	0.08 ± 0.07	0.06 ± 0.04	0.06 ± 0.04
24	0.57 ± 0.28	0.13 ± 0.07	0.06 ± 0.03	0.06 ± 0.05	0.05 ± 0.06	0.04 ± 0.02

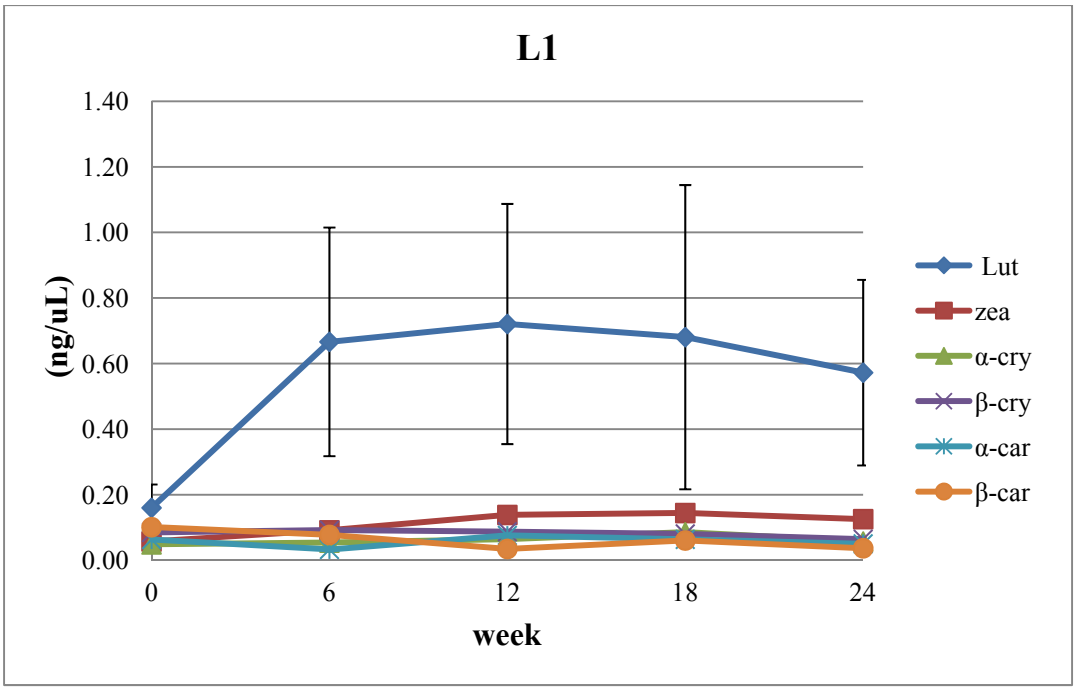


Figure 43: Comparison of carotenoids concentration in L1 group.
Numeric values are shown in table 5.

For lutein: $p < 0.001$, and for zeaxanthin: $p < 0.007$. There was a significant statistical difference when the subjects were compared to baseline. For the rest of the carotenoids: $p > 0.20$, no statistical difference exists when compared to baseline.

L2 group

Table 37: Carotenoid concentrations after supplementation in L2 (mean ± standard deviation).

week	Lut (ng/μL)	zea (ng/μL)	α-cry (ng/μL)	β-cry (ng/μL)	α-car (ng/μL)	β-car (ng/μL)
6	0.65 ± 0.37	0.08 ± 0.04	0.05 ± 0.02	0.11 ± 0.21	0.03 ± 0.02	0.06 ± 0.05
12	0.76 ± 0.58	0.16 ± 0.08	0.07 ± 0.02	0.13 ± 0.15	0.01 ± 0.01	0.05 ± 0.02
18	0.69 ± 0.60	0.12 ± 0.10	0.07 ± 0.04	0.11 ± 0.12	0.03 ± 0.01	0.06 ± 0.05
24	0.60 ± 0.40	0.09 ± 0.05	0.06 ± 0.03	0.10 ± 0.10	0.04 ± 0.02	0.10 ± 0.08

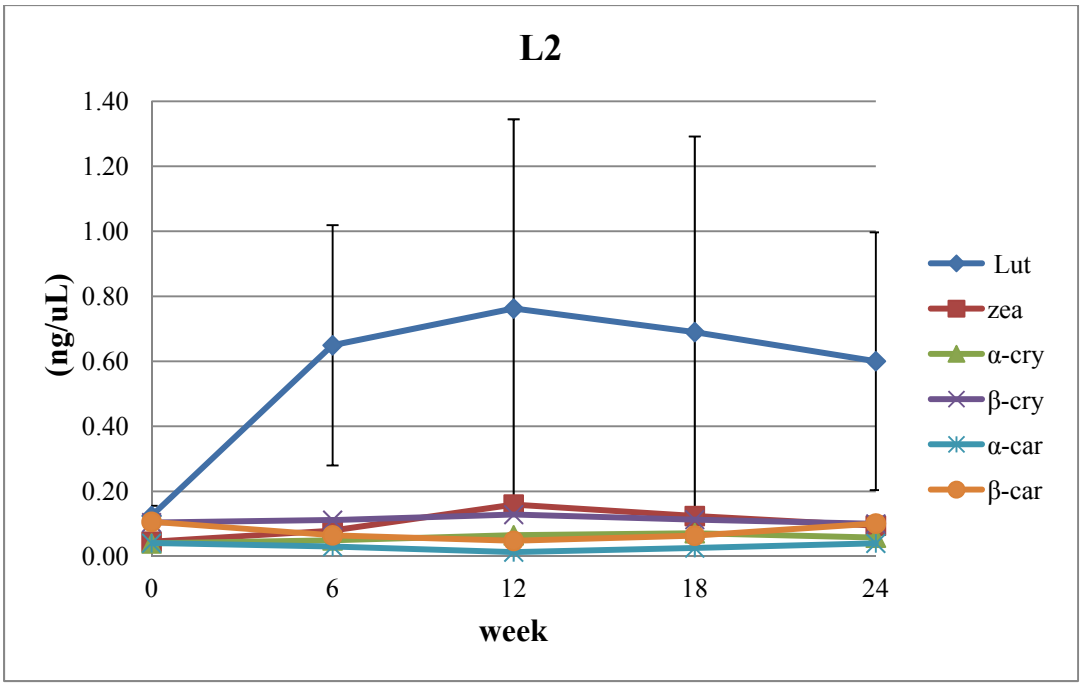


Figure 44: Comparison of carotenoids concentration in L2 group.
Numeric values are shown in table 6.

For lutein and for zeaxanthin: $p < 0.001$. There was a significant statistical difference when the subjects were compared to baseline. For the rest of the carotenoids (except for α-cryptoxanthin) : $p > 0.20$, no statistical difference exists when compared to baseline. For α-cryptoxanthin: $p > 0.04$.

MPOD results:

The MPOD rate for each study group is summarized in table 7:

Table 38: MPOD rates for each group (mean ± standard deviation) in study I.

	P1	**L1**	**L2**
Rate of MPOD increase (AU/day)	-0.0008 ± 0.0014	0.0017 ± 0.0011	0.0019 ± 0.0005

Study II:

P2 group (placebo)

Table 39: Carotenoid concentrations after supplementation in P2 (mean ± standard deviation).

week	Lut (ng/µL)	zea (ng/µL)	α-cry (ng/µL)	β-cry (ng/µL)	α-car (ng/µL)	β-car (ng/µL)
6	0.16 ± 0.08	0.07 ± 0.03	0.05 ± 0.04	0.11 ± 0.06	0.04 ± 0.04	0.05 ± 0.03
12	0.18 ± 0.05	0.08 ± 0.03	0.06 ± 0.04	0.13 ± 0.08	0.05 ± 0.01	0.04 ± 0.02
18	0.19 ± 0.10	0.08 ± 0.03	0.07 ± 0.06	0.13 ± 0.07	0.04 ± 0.00	0.05 ± 0.03
24	0.17 ± 0.07	0.08 ± 0.02	0.07 ± 0.07	0.12 ± 0.08	0.03 ± 0.01	0.05 ± 0.02

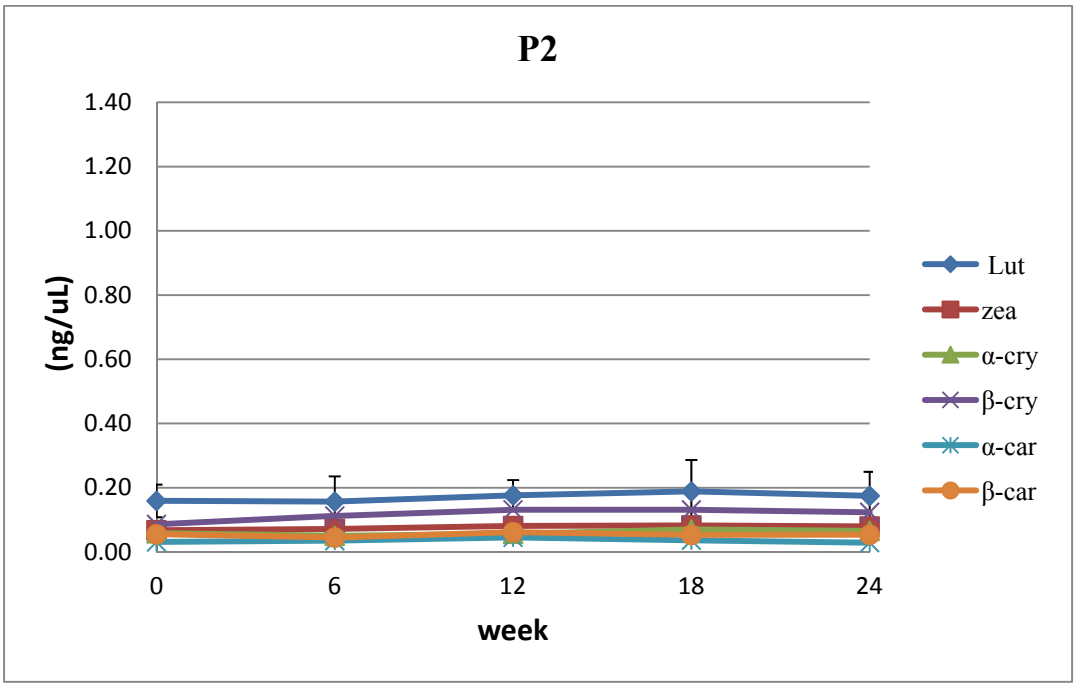


Figure 45: Comparison of carotenoids concentration in P2 group.
Numeric values are shown in table 7.

No statistical difference ($p > 0.10$) was found between the baseline and the response after supplementation for all the carotenoids in this group.

L3 group

Table 40: Carotenoid concentrations after supplementation in L3 (mean ± standard deviation).

week	Lut (ng/µL)	zea (ng/µL)	α-cry (ng/µL)	β-cry (ng/µL)	α-car (ng/µL)	β-car (ng/µL)
6	0.24 ± 0.11	0.07 ± 0.03	0.04 ± 0.01	0.12 ± 0.07	0.12 ± 0.10	0.09 ± 0.08
12	0.27 ± 0.18	0.08 ± 0.03	0.05 ± 0.04	0.10 ± 0.05	0.03 ± 0.00	0.05 ± 0.02
18	0.27 ± 0.09	0.10 ± 0.03	0.05 ± 0.02	0.11 ± 0.05	0.04 ± 0.04	0.07 ± 0.06
24	0.30 ± 0.13	0.10 ± 0.04	0.05 ± 0.02	0.12 ± 0.05	0.03 ± 0.00	0.05 ± 0.03

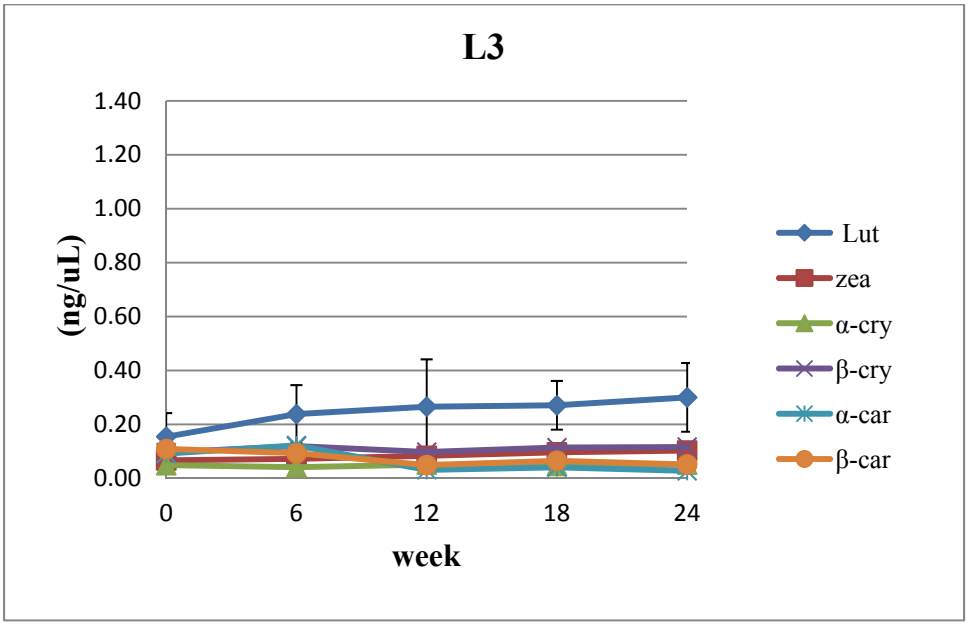


Figure 46: Comparison of carotenoids concentration in L3 group.
Numeric values are shown in table 8.

For lutein: $p < 0.004$, there was a significant statistical difference when the subjects were compared to baseline. For zeaxanthin: $p > 0.08$, and for the rest of the carotenoids: $p > 0.20$, no statistical difference exists when compared to baseline.

L4 group

Table 41: Carotenoid concentrations after supplementation in L4 (mean ± standard deviation).

week	Lut (ng/µL)	zea (ng/µL)	α-cry (ng/µL)	β-cry (ng/µL)	α-car (ng/µL)	β-car (ng/µL)
6	0.61 ± 0.22	0.10 ± 0.02	0.06 ± 0.03	0.10 ± 0.08	0.07 ± 0.02	0.09 ± 0.04
12	0.59 ± 0.39	0.10 ± 0.06	0.06 ± 0.03	0.11 ± 0.07	N.D.	0.07 ± 0.07
18	0.61 ± 0.23	0.12 ± 0.05	0.06 ± 0.03	0.12 ± 0.07	0.03 ± 0.00	0.09 ± 0.08
24	0.62 ± 0.40	0.13 ± 0.07	0.06 ± 0.02	0.12 ± 0.06	0.04 ± 0.01	0.05 ± 0.04

N.D.= no data were recorded.

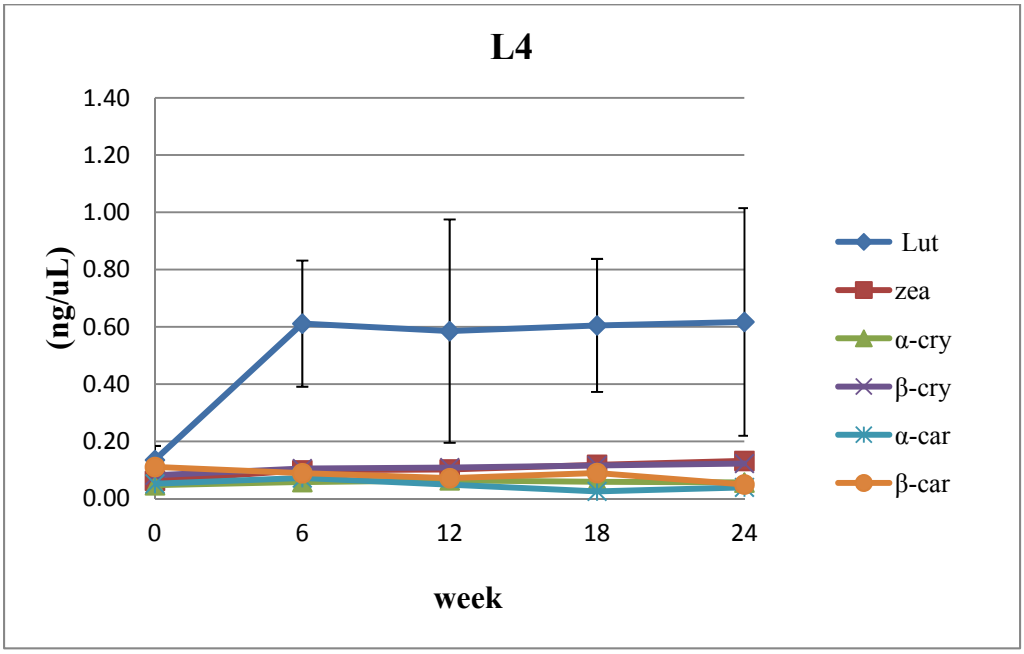


Figure 47: Comparison of carotenoids concentration in L4 group.
Numeric values are shown in table 9.

For lutein: $p < 0.001$, and for zeaxanthin: $p < 0.004$. There was a significant statistical difference when the subjects were compared to baseline. For the rest of the carotenoids: $p > 0.10$, no statistical difference exists when compared to baseline.

MPOD results:

The MPOD increase for each study group is summarized in table 11:

Table 42: MPOD rates for each group (mean ± standard deviation) in study II.

	P2	L3	L4
Rate of MPOD increase (AU/day)	0.0011 ± 0.0008	0.0011 ± 0.0012	0.0026 ± 0.0006

There is no difference between the placebo group and the L3 group.

3.4. Discussion:

3.4.1. Study I:

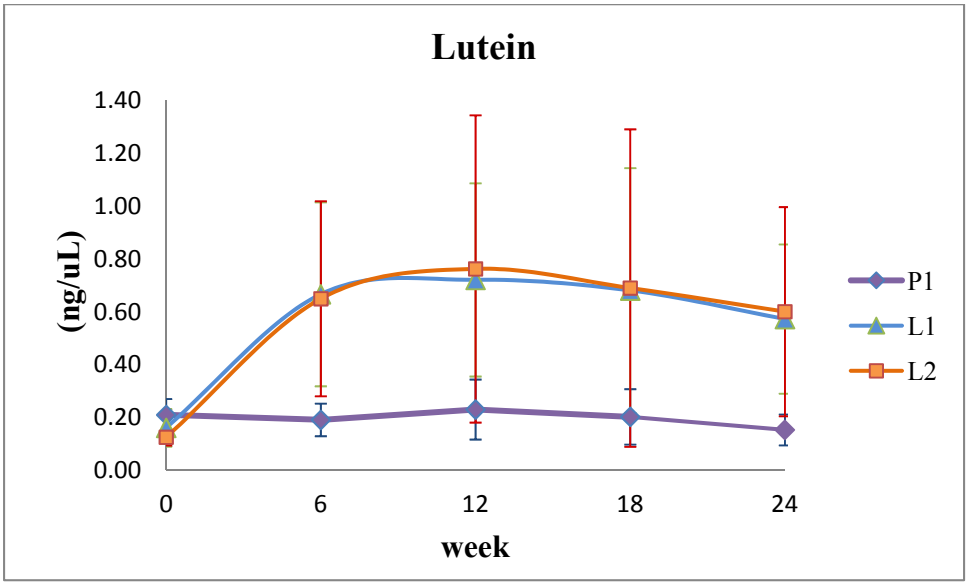


Figure 48: Comparison of lutein concentration in serum for study I.

Figure 48 shows a comparison for the lutein concentration in serum between the three groups. According to the values during the supplementation period, the concentration of lutein in

the case of the supplemented groups increases within the first 6 weeks and remains almost constant for the rest of the study (until week 24th is completed). It reached its maximum value during the 12th week of supplementation for both L1 and L2. The placebo group remains constant as expected. The error bars in both cases (L1 y L2) are more than 50% of the average of the concentration value in each period of time. This variability is caused to the differences of the concentration of lutein in serum for each subject. Lutein concentrations vary from one subject to another as a consequence of their diet. The ANOVA run for the supplemented groups for the average concentration of lutein in serum during supplementation demonstrates that the results for L1 and L2 are highly significant different ($p<0.0002$) from baseline values.

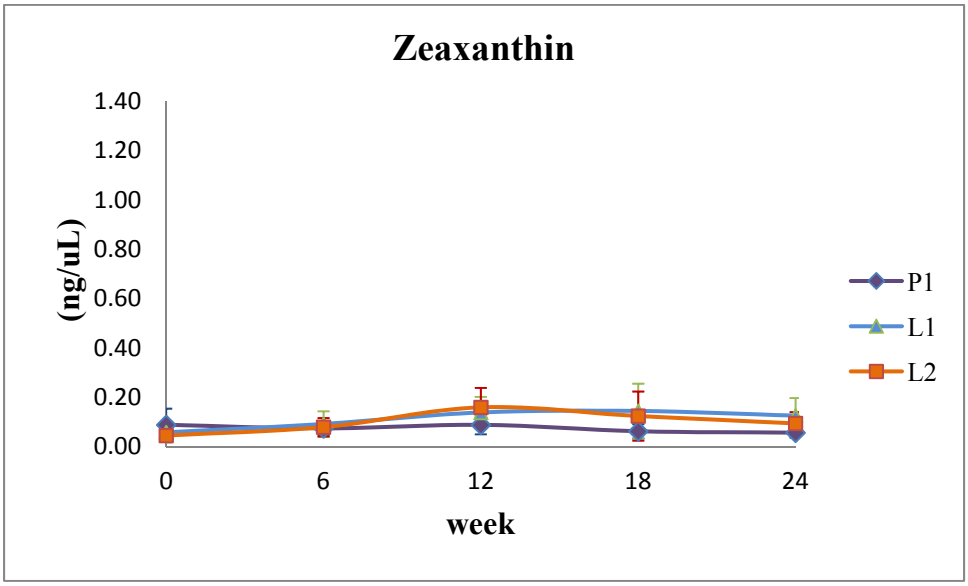


Figure 49: Comparison of zeaxanthin concentration in serum for study I.

Figure 49 shows the comparison for zeaxanthin in the study. The concentrations are smaller since the ratio L/Z in human serum is 2- 3. For the ANOVA run like in the case of lutein the results were: L1 $p<0.006$, and for L2 $p<0.0005$, as similar to the lutein case shown above the

error is around 50% or more. Again, is because of the variability of the subjects within the groups.

Table 43 summarizes the average concentration for each group after supplementation (weeks 6, 12, 18 and 24) in comparison with the baseline concentrations for lutein and zeaxanthin.

Table 43: Concentrations for lutein and zeaxanthin in each group before and after supplementation in study I.

	Lutein (ng/μL)			**Zeaxanthin** (ng/μL)		
	P1	L1	L2	P1	L1	L2
Baseline	0.21 ± 0.06	0.16 ± 0.07	0.12 ± 0.03	0.09 ± 0.07	0.06 ± 0.04	0.05 ± 0.01
Average	0.19 ± 0.02	0.66 ± 0.05	0.68 ± 0.10	0.07 ± 0.01	0.13 ± 0.02	0.11 ± 0.02

In Figure 50 we can see a comparison of the average concentrations for lutein and zeaxanthin after supplementation by groups. The increase of lutein for group L1 is around four times more, and for group L2 is more than five times of the corresponding to the baseline level. This high increase can be attributed to the fact that the supplementation given was of 20mg of free lutein in each case (L1 and L2). The only difference between them is the formula used for each pill. For zeaxanthin the amounts double the amount of the baseline.

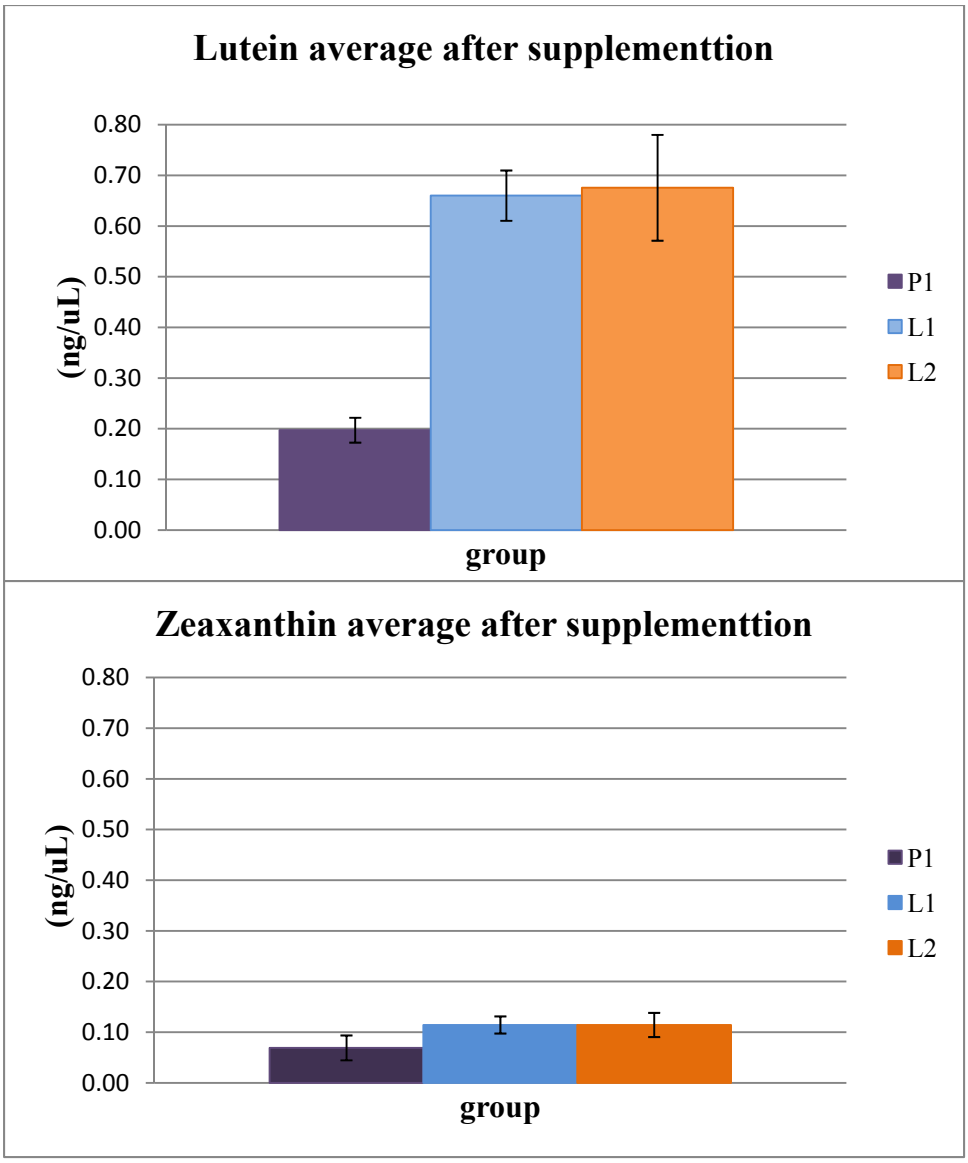


Figure 50: Average concentrations for lutein and zeaxanthin after supplementation in serum for study I.

The rate of change of MPOD was determined as the slope of a linear fit to the MPOD measures during the 24 weeks of supplementation (see table 38) and fixed to baseline of each group.. The standards deviation of the MPOD mean rate for groups L1 and P1 are larger (more than 60%) than for group L2 (approximately 25%). This numbers evidence the variability

between subjects in the groups. The levels of MPOD are smaller in comparison with the serum response. The correlation between the average serum levels after supplementation and MPOD rates is almost perfectly linear (R^2=0.9997) for this study, but no significant statistical difference between the groups was found.

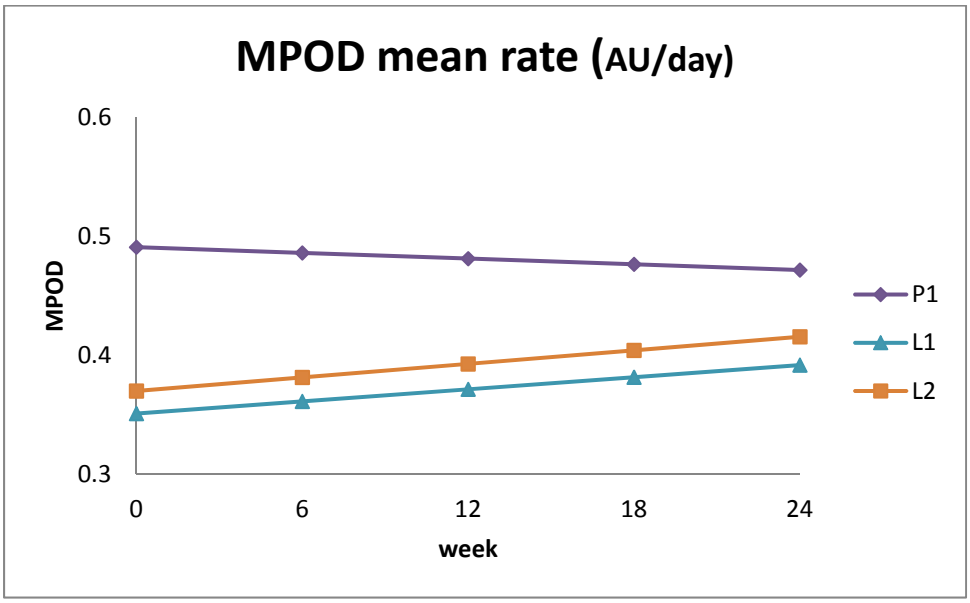


Figure 51: Comparison of mean rate of MPOD for study I.

3.4.2. Study II:

Figure 52 illustrate the comparison of lutein concentration in serum between the study groups. The placebo group remains fairly constant along the study period. As seen in the first study, the concentration of lutein in serum for the supplemented groups increases during the first 6 weeks and remains slightly constant for the next 18 weeks. For the L4 group the maximum value is reached at week sixth and the value remains constant after. For the L3 group the concentration continued increasing approximately 12% every six weeks after the sixth week.

Once again, the error bars appear to be around 50% for the supplemented groups because of the variability within the subjects. The ANOVA run for group L3 ($p < 0.004$) and for L4 ($p < 0.0002$) evidence that the results for the lutein concentrations are statistically different form the baseline values.

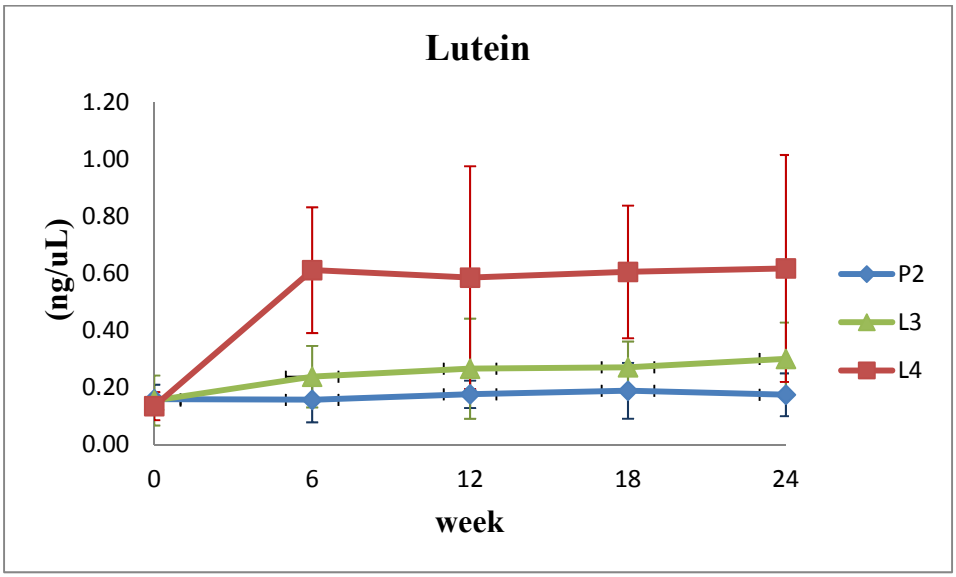


Figure 52: Comparison of lutein concentration in serum for study II.

Figure 53 (see below) shows the comparison for the zeaxanthin concentration between the groups. The concentrations as seen in the first study are lower than the ones for lutein. But in this case, for group L3 the ANOVA run ($p > 0.08$) indicates that there is not a statistically difference from the baseline. In the case of L4 group the ANOVA indicate that there is a significant difference with the baseline values ($p < 0.005$).

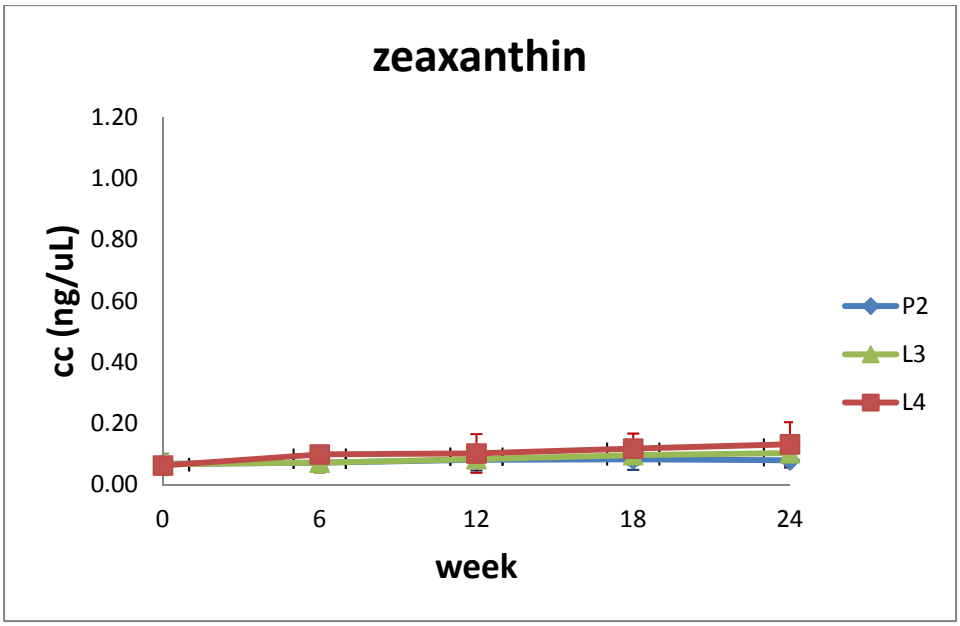


Figure 53: Comparison of zeaxanthin concentration in serum for study II.

Table 44 summarizes the average concentration for each group after supplementation (weeks 6, 12, 18 and 24) in comparison with the baseline concentrations for lutein and zeaxanthin.

Table 44: Concentrations for lutein and zeaxanthin in each group in study II.

	Lutein (ng/µL)			**Zeaxanthin** (ng/µL)		
	P2	L3	L4	P2	L3	L4
Baseline	016. ± 0.05	0.15 ± 0.09	0.13 ± 0.05	0.07 ± 0.02	0.07 ± 0.04	0.06 ± 0.02
Average	0.17 ± 0.01	0.27 ± 0.03	0.60 ± 0.08	0.08 ± 0.00	0.09 ± 0.00	0.11 ± 0.02

In Figure 54 we can see a comparison of the average concentrations for lutein and zeaxanthin after supplementation by groups. The increase of lutein for group L3 is almost two times higher, and for group L4 is of almost 5 times higher than the corresponding to the baseline level. The increase in this case is higher for the L4 group than for the L3 group. Although the

supplementation level was of 9mg/day in this case for both groups, L4 supplemented formula results to be superior to the formula use for L3. We have to highlight the fact that the formula for L4 includes other vitamins. For zeaxanthin L4 increases in almost two times the amount of the baseline. While for L3 the increase is not significant.

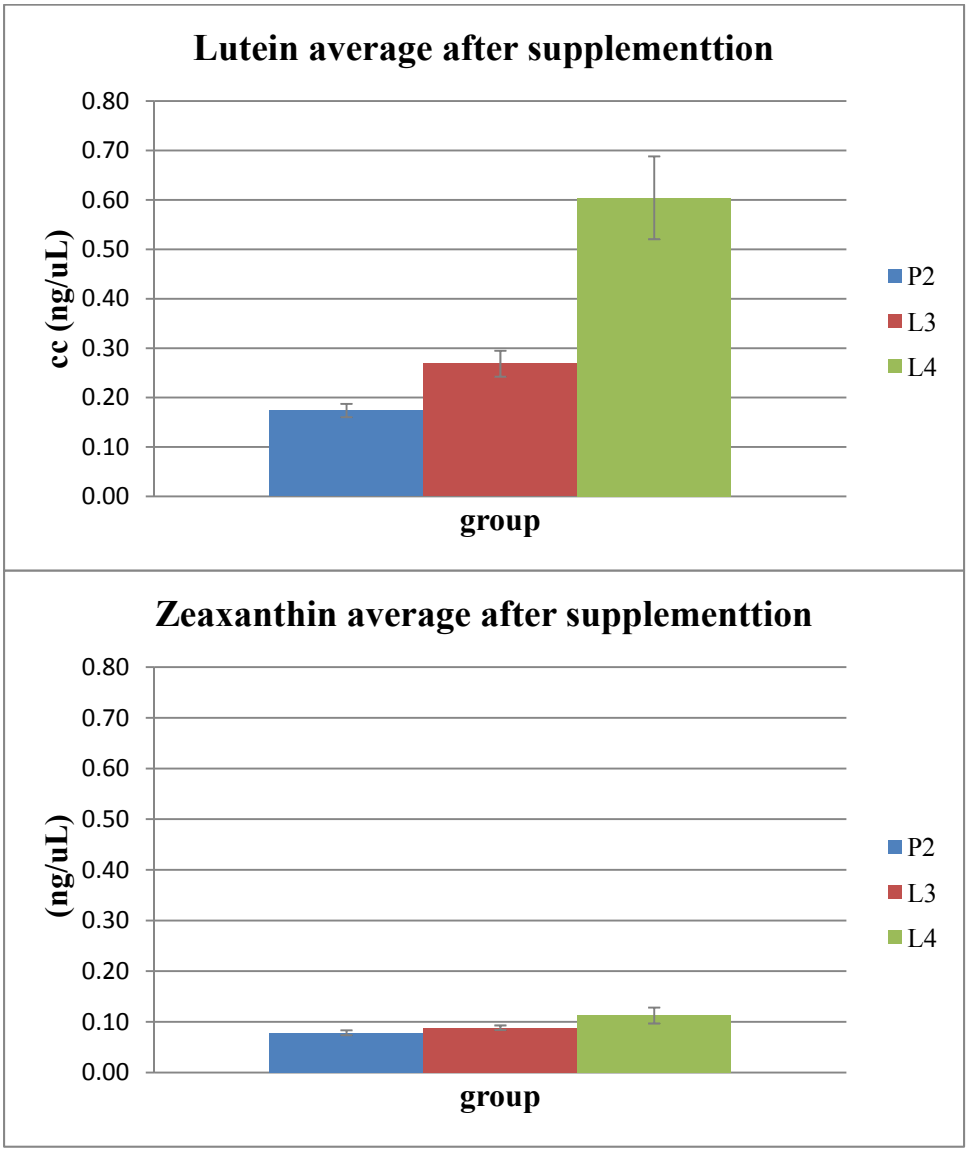


Figure 54: Average concentrations for lutein and zeaxanthin after supplementation in serum for study II.

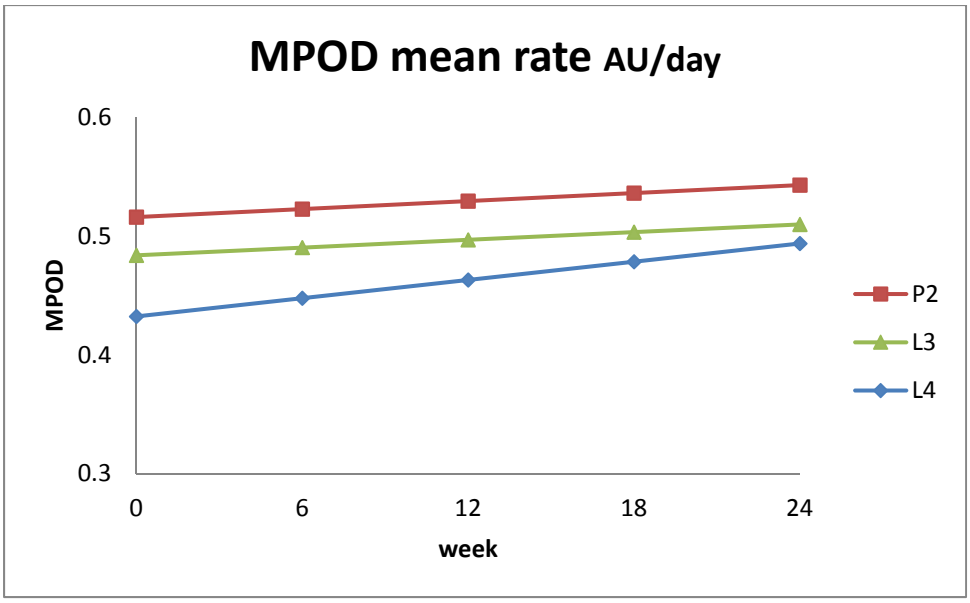


Figure 55: Comparison of mean rate of MPOD for study II.

The rate of change of MPOD was determined as the slope of a linear fit to the MPOD measures during the 24 weeks of supplementation (see table 42) and fixed to baseline of each group. The MPOD mean rate for groups P2 and L3 has similar slopes; therefore there is no difference of the means between these two groups. The variability of the MPOD mean rate for groups P2 and L3 are larger (more than 70%) than for group L4 (23%). There is a statistical difference between the baseline and the average of the MPOD levels for the subjects in group L4. Although the same amount of lutein was supplied to both groups: L3 and L4; there is a difference in the response.

3.5. Conclusion

According to the data collected in this study from 61 healthy subjects, the concentration levels of β-carotene, α-carotene, β-cryptoxanthin, and α-cryptoxanthin remain unaltered in human serum during the 24 weeks of study. No significant statistical difference was found in each group when the concentrations of the mentioned carotenoids that were studied were compared with the

baseline levels in intervals of six weeks after supplementation. The absorption of the major carotenoids in the human serum was not affected by the intake of lutein supplement pills. Therefore, we can infer that the biological functions of these carotenoids are not altered, either. Lutein supplementation has no effect on these major carotenoids in the human serum, with the exception of lutein and zeaxanthin.

For the supplemented groups, the lutein concentration in human serum increased during the 24 weeks in both studies. The concentrations of lutein and zeaxanthin in the placebo groups remained at baseline levels during the study. In study I, subjects were given a 20 mg/day dose of free lutein. Both groups showed a positive statistical difference on the average lutein concentration after supplementation, reaching up to a five-fold increase. For zeaxanthin, the average concentration was doubled. In study II, subjects were given a 9 mg/day dose of free lutein. Only the group L4 reached similar concentration levels for lutein and zeaxanthin, as seen in study I. Although the same dose was supplied for group L3, the lutein concentration was only doubled and the zeaxanthin concentration levels did not differ from the baseline. In this case, we can conclude that the formulation used for L3 pills is not reliable. However, it should be taken into account that the formulation of L4 includes other vitamins that might help to improve the absorption of lutein in the body. Further studies have to be made to prove this idea. Although variability in the concentrations of lutein and zeaxanthin were observed when measured every six weeks, most probably due to the diet of the subjects, there was a good response when 9 mg – 20 mg/day of lutein were supplied.

The MPOD increased in the supplemented groups L1, L2, and L4, during the study. However, differences in MPOD rates of increase between the groups did not reach a statistical significant difference. For the placebo group P1, there is no difference when compared to the baseline values, as was expected. The groups P2 and L3 had similar values of MPOD rate of

increase, and there was no difference in the MPOD values when we compared them to their baselines, or between the groups. This fact agrees with the previous statement that L3 did not produce a significant increase in the lutein and zeaxanthin concentration levels. The increase of the MPOD response for the subjects in groups L1, L2, and L4 is slower in comparison with the increase of lutein concentration in the serum. The absorption of lutein and zeaxanthin in the bloodstream is faster than the transport of those carotenoids to the macula. The transport is associated with a selective protein-binding mechanism. This may explain the difference in the rate of increase of levels in serum and in MPOD.

Studies have showed that the consumption of lutein is low in a normal human diet. In this study, we showed that lutein supplementation does not influence the levels of the other major carotenoids in the human serum. The concentration levels of lutein and zeaxanthin increased during supplementation. The increase of these two carotenoids can lead to increased macular pigment density in the eye, which helps to protect the retina from photoxidation and lower the risk of macular degeneration. Therefore, we can suggest that lutein is a good supplementation to be included in a daily diet.

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