DEPARTMENT OF HEALTH & HUMAN SERVICES

Public Health Service
Food and Drug Administration

Memorandum

Date: June 13, 2001

From: Director, Division of Standards and Labeling Regulations, Office of Nutritional Products, Labeling and Dietary Supplements, HFS-820

Subject: 75-Day Premarket Notification for New Dietary Ingredients

To: Dockets Management Branch, HFA-305

New Dietary Ingredient:	zeaxanthin
Firm:	Roche Vitamins, Inc.
Date Received by FDA:	March 22, 2001
90-Day Date:	June 20, 2001

In accordance with the requirements of section 413(a) of the Federal Food, Drug, and Cosmetic Act, the attached 75-day premarket notification for the aforementioned new dietary ingredient should be placed on pubic display in docket number 95S-0316 **after** June 20, 2001.

Felicia B. Satchell

Felicia B. Satchell

95S-0316

RPT 96

DEPARTMENT OF HEALTH AND HUMAN SERVICES

Public Health Service

Food and Drug Administration
Washington, DC

APR 17 2001

A. Davidovich, D.V.M., Ph.D., D.A.B.T.
Associate Director, Regulatory Affairs
Roche Vitamins Inc.
45 Waterview Boulevard
Parsippany, New Jersey 07054-1298

Dear Dr. Davidovich:

This is to inform you that the notification, dated March 21, 2001, you submitted pursuant to 21 U.S.C. 350b(a)(2) was received and filed by the Food and Drug Administration (FDA) on March 22, 2001. Your notification concerns the substance called Zeaxanthin that you assert is a new dietary ingredient. This notification is a resubmission of a notification dated August 3, 2000 for the same ingredient that FDA responded to in a letter dated October 19, 2000.

In accordance with 21 C.F.R § 190.6(c), FDA must acknowledge its receipt of a notification for a new dietary ingredient. For 75 days after the filing date (i.e., after June 5, 2001), you must not introduce or deliver for introduction into interstate commerce any dietary supplement that contains Zeaxanthin.

Please note that acceptance of this notification for filing is a procedural matter and thus, does not constitute a finding by FDA that the new dietary ingredient or supplement that contains the new dietary ingredient is safe or is not adulterated under 21 U.S.C. 342. As another procedural matter, your notification will be kept confidential for 90 days after the filing date. After June 20, 2001, the notification will be placed on public display at FDA's Docket Management Branch in docket number 95S-0316. However, any trade secret or otherwise confidential commercial information in the notification will not be disclosed to the public.

Please contact us at (202) 205-4168, if you have any questions concerning this matter.

Sincerely yours,

Rhonda R. Kane, M.S., R.D.
Consumer Safety Officer
Dietary Supplements Team
Division of Standards
and Labeling Regulations
Office of Nutritional Products, Labeling
and Dietary Supplement
Center for Food Safety
and Applied Nutrition

March 21, 2001

VIA COURIER

Roche

Vitamins

Division of Standards and Labeling Regulations
Office of Nutritional Products, Labeling, and Dietary Supplements (HFS-820)
Center for Food Science and Applied Nutrition
Food and Drug Administration
200 C St. SW
Washington, DC 20204

Re: New Dietary Ingredient Notification: Zeaxanthin

Dear Sirs:

Enclosed please find an original and three copies of the "Notification of the Marketing of a New Dietary Ingredient: Zeaxanthin", submitted pursuant to section 413 of the Federal Food, Drug and Cosmetic Act.

Please note that pursuant to 21 C.F.R. § 20.61, Roche Vitamins Inc. designates as confidential Section 2.1.3 " Manufacturing Principles", Chapter 5 "Toxicology" and Volumes V-VI-VII, containing a final report and related materials for a toxicity study.

Zeaxanthin is a carotenoid found in the retina (macula) of humans. Because humans cannot synthesize carotenoids, food is the only source, particularly from consumption of yellow/orange/ red fruits and dark green leafy vegetables. Roche synthetic zeaxanthin is identical to natural zeaxanthin. Roche Vitamins Inc. will sell Zeaxanthin 5% TG to either manufacturers of dietary supplements or through distributors. Roche Vitamins Inc. will not sell the product to retail customers.

This notification is divided into seven volumes. We have included all the published references; they are organized by chapter, in alphabetical order and divided into four volumes (Volumes I, II, III, IV):

VOLUME I	Notification text	
	Appendix 1	References (published) Chapter 3 (Alpern - Ham)
VOLUME II	Appendix 1 (continued)	References (published) Chapter 3 (Hammond - Sanders)
VOLUME III	Appendix 1 (concluded)	References (published) Chapter 3 (Schalch - Zorge)
	Appendix 2	References (published) Chapter 4 (Botterweck - Khachik)
VOLUME IV	Appendix 2 (concluded)	References (published) Chapter 4 (Kull - Yuan)
	Appendix 3	References (published) Chapter 5
	Appendix 4	References (published) Chapter 6
VOLUME V	Comprehensive overview on Eye Examination (One year monkey study)	
	One year monkey study	
VOLUME VI	One year monkey study (continued)	
VOLUME VII	One year monkey study (concluded)	
	Amendments to final report No1, 2, 3	

Thank you for your attention to this matter. If you have any questions regarding this notification, please do not hesitate to call me at the number below.

Sincerely,

A. Davidovich, DVM, PhD, DABT
Associate Director Regulatory Affairs

Enclosure

Tel: 973 257-8326
Fax: 973 257-8414

955-0316

RPT

Roche Vitamins Inc. 45 Waterview Boulevard
Parsippany, New Jersey 07054-1298

VOLUME I

Notification

Appendix 1: References for Chapter 3 (Alpers – Ham)

Zeaxantin

A Dietary Ingredient for Dietary Supplements

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VOLUME IV

Appendix 2 (concluded): **References for Chapter 4 (Kull – Yuan)**

Appendix 3: **References (published) for Chapter 5**

Appendix 4: **References for Chapter 6**

VOLUME V

Comprehensive Overview On Eye Examinations
One Year Monkey Study Report

VOLUME VI

One Year Monkey Study Report (continued)

VOLUME VII

One Year Monkey Study Report (concluded)
- Amendment to Final Report No.1
- Amendment to Final Report No.2
- Amendment to Final Report No.3

Zeaxanthin, a Dietary Ingredient for Dietary Supplements

Roche Vitamins Incorporated

1.1 Name and Address of the Distributor

The distributor of zeaxanthin is the company:

Roche Vitamins Inc.
45 Waterview Blvd.
Parsippany, NJ 07054-1298
USA
Contact: A. Davidovich, DVM, PhD
Associate Director Regulatory Affairs
Telephone: (973) 257 8325
Fax: (973) 257 8414

Zeaxanthin is manufactured for Roche Vitamins Inc. by

F. Hoffmann-La-Roche AG
Postfach
CH-4070 Basel
Switzerland
Phone *41-61/688 11 11
Fax. *41-61/691 93 91

1.2 Name of the Dietary Ingredient for Dietary Supplements

The dietary ingredient is zeaxanthin.

The product will be available as a bulk ingredient in a beadlet formulation containing 5% zeaxanthin (Zeaxanthin 5%TG), for the manufacture of dietary supplements.

Roche Vitamins Inc. will sell Zeaxanthin 5% TG to either manufacturers of dietary supplements or through distributors . Roche Vitamins Inc. will not sell this product to retail customers.

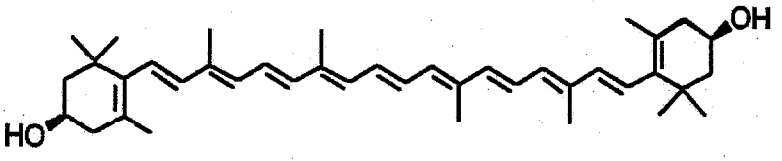
Chapter 2: Description of Zeaxanthin, the Dietary Ingredient

2.1 Chemistry, Manufacturing and Controls

2.1.1 Chemistry

Zeaxanthin is a dietary ingredient for dietary supplements, which belongs to the class of carotenoids called xanthophylls. Zeaxanthin has limited solubility in lipids and it is practically insoluble in water.

The chemical structure is:



Zeaxanthin

The chemical composition of zeaxanthin is $C_{40}H_{56}O_2$ and the molecular weight is 568.89. The CAS Number is 144-68-3.

Three isomers of zeaxanthin are normally present in the retina of which the most abundant isomer is (3R,3'R)-zeaxanthin. In addition, two minor isomers are also found: (3R,3'S)-(meso)-zeaxanthin and (3S,3'S)-zeaxanthin. In the retina the ratio of (3R,3'R):(3R,3'S):(3S,3'S) is between approximately 3:2.5:1 and 14:4:1 depending on the region. The inner region contains relatively more (3R,3'R) than the center and outer regions.

The presently described dietary ingredient contains almost exclusively (3R,3'R)-zeaxanthin, as well as very small amounts of (3R,3'S)-(meso)-zeaxanthin and (3S,3'S)-zeaxanthin. The beadlet form contains about 98 % (3R,3'R), 2 % (3R,3'S) and 0.1 % (3S,3'S).

2.1.2 Assay and Specifications

Zeaxanthin 5% TG (beadlet formulation) has a minimum guaranteed content of 5% pure zeaxanthin. Pure zeaxanthin is a reddish powder, which is finely dispersed in a cornstarch-coated matrix of gelatin and sucrose. Ascorbyl palmitate, sodium ascorbate and *dl*-α-tocopherol are added as antioxidants. The 5% TG formulated zeaxanthin product appears as reddish, free-flowing beadlets with the following composition in 1 g:

2.1.4. Stability and Use of Zeaxanthin

2.1.4.1 Stability

Zeaxanthin 5% TG is sensitive to heat, light and humidity. The product is considered to be stable for at least 18 months based on previous experience with related compounds with similar formulations. Stability tests are in progress, and the ongoing testing after 6 months shows 100% stability at temperatures up to 35°C. It is recommended that the product may be stored at a temperature below 15°C for up to 18 months.

2.1.4.2 Recommended Daily Intake (Proposed Use).

A recommended daily intake of 1 mg of Zeaxanthin /day is proposed.

2.2 Proposed Labeling

Zeaxanthin is a dietary supplement that helps maintain healthy eyesight. This statement has not been evaluated by the Food and Drug Administration. This product is not intended to diagnose, treat, cure, mitigate or prevent any disease.

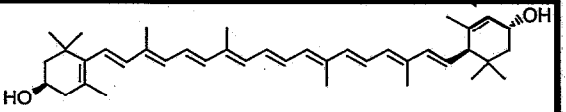
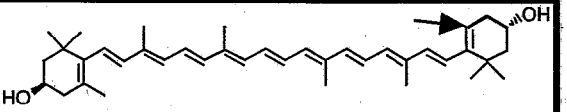
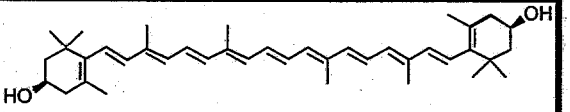
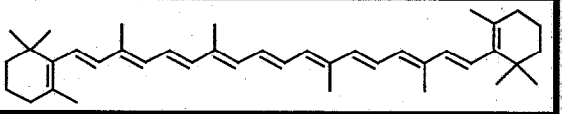
Chapter 3: Biological Relevance of Zeaxanthin

3.1 Introduction

Three yellow carotenoids 3R,3'R-zeaxanthin, 3R,3'S (meso)-zeaxanthin, and lutein (Table 1) are the predominant constituents of the macula lutea, as the yellow spot in the center of the human retina is called (Nussbaum, 1981; Schalch et al., 1999). In the center of the macula, the concentration of zeaxanthin and lutein is estimated to be around 1 mM. This is three orders of magnitude higher than typical carotenoid concentrations in other human tissues (Landrum et al.,

Table 1

The macular carotenoids in comparison to β-carotene: typical concentrations in human plasma and amounts in specific areas of the retina. The arrows indicate how the chemical structures of lutein and meso-zeaxanthin differ by virtue of the position of a double bond.

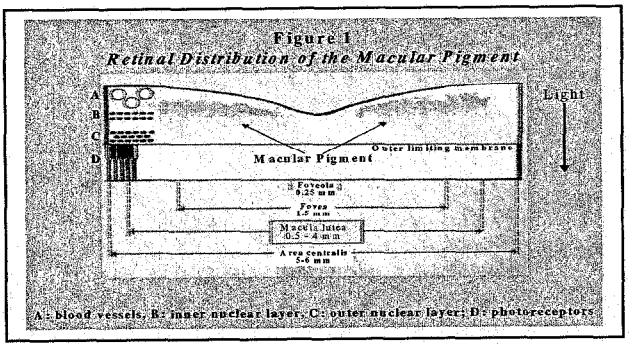
Carotenoid	Plasma concentration (μmol/L)	Content in retinal areas[4] (pmoles/mm²)	Chemical structure
Lutein	0.29[1] 0.19[2] 0.28[3]	inner: 2.4 medial: 0.22 outer: 0.065	
Meso-zeaxanthin	None	inner: 1.4 medial: 0.037 outer: 0.0061	
Zeaxanthin	0.04[1] 0.06[2] 0.07[3]	inner: 1.7 medial: 0.094 outer: 0.020	
β-carotene	0.22[2] 0.46[3]	None	

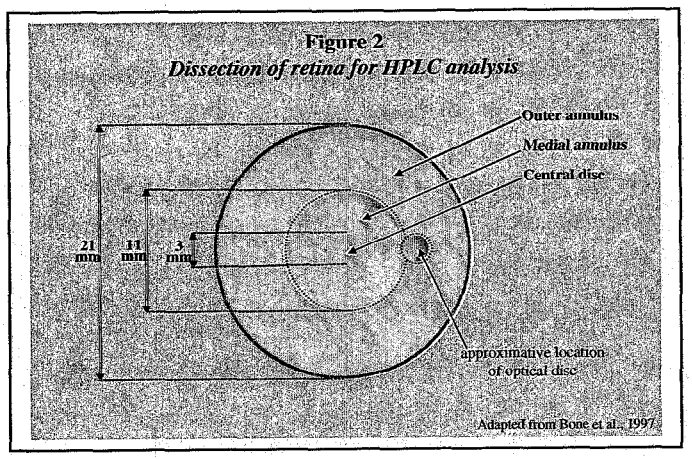
[1]: Khachik et al., 1997; [2]: Olmedilla et al., 1997a; [3]: Ascherio et al., 1992; [4]: Landrum et al., 1999.

1999), making the macula lutea the most conspicuous accumulation of carotenoids in the human body. This specific accumulation of carotenoids in the macula has led to increased interest in whether lutein and zeaxanthin intake plays a role in reducing the risk of age-related macular degeneration (AMD), the most frequent cause of irreversible vision loss in the US.
Because one of the macular carotenoids, lutein, has been reviewed extensively, this chapter will focus on the unique properties of zeaxanthin as well as the properties that it shares with lutein.

3.2 Occurrence and distribution of zeaxanthin and lutein in retina and macula - zeaxanthin is a major constituent of the center of the macula

The presence of the yellow macular pigment in the center of the retina (the macula lutea or yellow spot) has been known since the late 18th century. After intensive research, it turned out that the occurrence of 3R,3'R-zeaxanthin, 3R,3'S-(meso)-zeaxanthin and lutein in the retina is very specific. None of the other plasma carotenoids is present in the retina. In particular, the non-polar carotenoids β-carotene and lycopene, that are normally found in substantial concentrations in the blood do not normally occur in the retina (Handelman et al., 1988). Canthaxanthin was the only other polar carotenoid that has been identified in the retinas of persons who had ingested elevated amounts of it for oral tanning purposes or to treat light sensitivity disorders.





Macular pigment is localized in Henle's fibres, the axons of the photoreceptors in the vicinity of the inner nuclear layer (B in Figure 1) (Snodderly et al., 1984 a & b), providing a very appropriate location to directly shield the photoreceptors from incoming blue light. While the macroscopic visibility of the yellow color marks just the regions of highest concentration of the macular pigment at and around the center of the retina, lutein and zeaxanthin also do occur in more peripheral regions of the retina, however in substantially lower concentrations than in the center.

Our knowledge of the detailed distribution of the macular carotenoids in the retina was established by the work of Bone et al. (1997) and further expanded by Landrum et al. (1999). They dissected human retinas using three trephines with diameters of 3, 11, and 21 mm (Figure 2). This produced a central disc containing most of the yellow spot ("central" area) and two concentric annuli, of which one is in the middle ("medial" area) and the other contains the peripheral retina ("outer" area). These areas were analyzed by HPLC to quantify the individual carotenoid molecules.

As can be seen from Table 1 and Figure 3, the highest concentrations of lutein, dietary zeaxanthin (3R, 3'R) and meso-zeaxanthin (3R, 3'S), a zeaxanthin isomer not found in food, are found in the fovea while lower concentrations are found in regions distal to the fovea. The relative abundance of lutein increases and meso-zeaxanthin decreases as distance from the foveola increases (Figure 3). The high concentration of zeaxanthin relative to lutein in the inner

retina is remarkable when contrasted against plasma values: lutein concentrations are 700% higher than 3R, 3'R-zeaxanthin in the plasma but only 40% higher in the inner retina (Table 1).

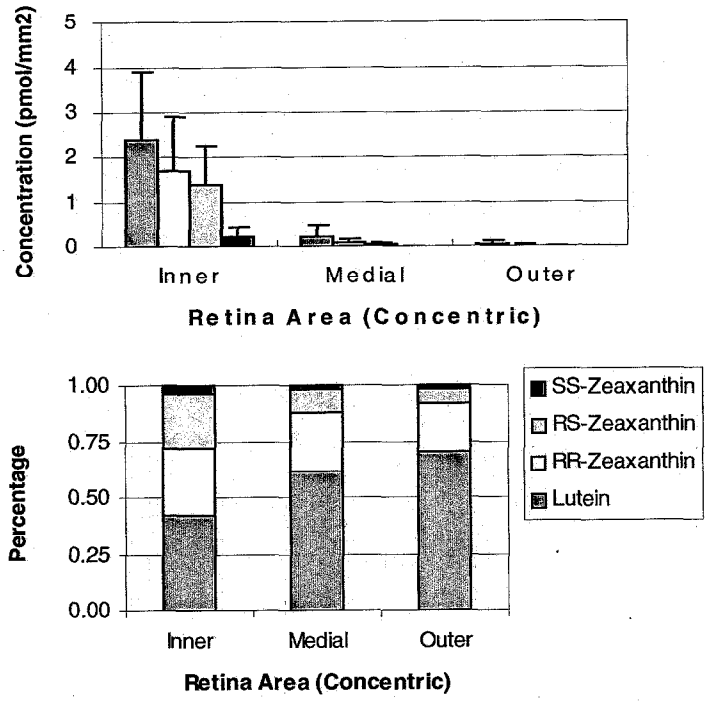
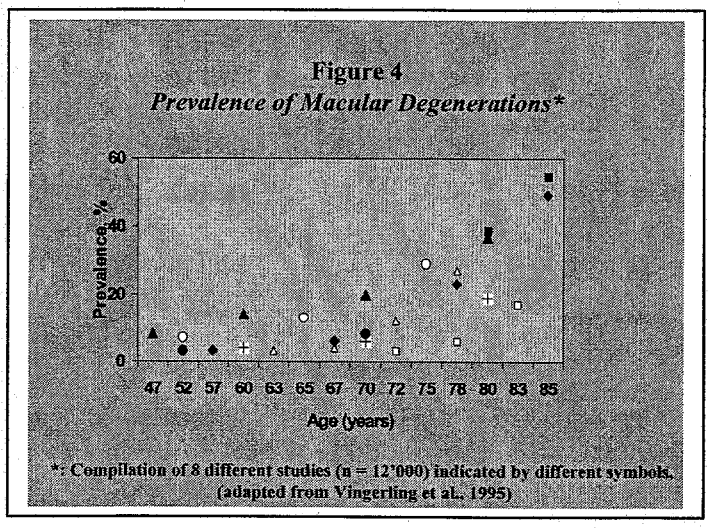


Figure 3: Absolute and relative concentrations of lutein, RR-zeaxanthin, RS-meso-zeaxanthin and SS-zeaxanthin in 16 human retinas (Landrum et al., 1999)

On the basis of this specific distribution pattern of the macular carotenoids, a hypothesis was put forward by Bone et al. (1997) that lutein may be preferentially associated with rods and zeaxanthin with cones. As mentioned earlier, one macular carotenoid, 3R,3'S-(meso)-zeaxanthin does not occur to any substantial amount in plasma nor in food. Therefore it was further hypothesized that this molecule either is transported into the retina with a very high efficiency or it must have be formed within the retina, presumably from lutein by enzymatic or photochemical processes that effect the migration of the isolated double bond of the lutein molecule into conjugation within the generated 3R,3'S-(meso)-zeaxanthin molecule (Table 1).

The exact mechanisms of uptake of lutein and zeaxanthin into the retina that would explain their specific distribution pattern have remained elusive, and the existence or non-existence of binding proteins that are instrumental in this uptake process is still unclear. Bernstein et al. (1997) had suggested that retinal tubulin may be the carotenoid-binding principle in the retina. However, the lack of specificity of this substance suggests that it may only passively stabilize zeaxanthin and lutein in the fovea probably in a similar way as actin stabilizes the carotenoid astaxanthin in salmon muscle. In a preliminary report, the same group reported the purification of a 28 kDa membrane-associated protein from human retinas that has xanthophyll-binding properties and apparently a somewhat higher affinity towards lutein than towards zeaxanthin (Balashov-Katz, Moore and Bernstein, 1999).

3.3 Age-related macular degeneration (AMD)



Age-related macular degeneration (AMD) is a multifactorial degenerative disease of the central part of the retina and the retinal pigment epithelium that manifests itself in an atrophic ("dry") and a neovascular ("wet") form (Campochiaro, 1999). The latter form is characterized by the presence of fluid accumulation with a gradual loss of central high-acuity vision due to hemorrhagic maculopathy. Ultimately this decline in visual acuity can lead to absolute loss of vision, and AMD is the leading cause of irreversible blindness in the US. Vingerling et al. (1995) have compiled data from eight epidemiological studies reporting prevalence data from more than 12,000 individuals of five countries. As can be seen in Figure 4, the prevalence of macular degeneration increases drastically from about 65 years of age and is becoming a more frequent ailment of the aging population.

The etiology of AMD is only poorly understood, and both genetic and environmental factors have been hypothesized as playing a role. One of the environmental factors seems to be ocular exposure to sunlight (McCarty & Taylor, 1999) in particular a history of exposure to blue light in the preceding 20 years (Taylor et al., 1992). In the presence of photosensitizers, that are abundant within the retina and pigmented epithelium, blue light can induce the formation of reactive oxygen species including singlet oxygen and superoxide radicals that in turn mediate oxidative damage. Furthermore, such damage can also be initiated independently from light by endogenous metabolic processes. The reactive oxygen species generated ultimately induce peroxidation of polyunsaturated fatty acids, in particular of docosahexanoic acid, a major lipid constituent of photoreceptor outer segments (Stone et al., 1979). Through such damage, the integrity of the complex of photoreceptors and the RPE (retinal pigment epithelium) is impaired and concomitantly the cyclic process of photoreceptor phagocytosis and renewal. Ultimately this can lead to the accumulation of cell debris and lipofuscin in Bruch's membrane, the formation of drusen and potentially neovascularization and retinal detachment. The retina is highly active metabolically and has a much higher blood flow than other tissues. Exposed to the simultaneous presence of light and oxygen (Schalch, 1992), the retina has an obvious need for antioxidant protection. At present, there is no cure for AMD other than laser treatment of the neovascular form with unsatisfactory results (RAD Study Group, 1999), and potentially photodynamic therapy with verteporfin (Bressler & Bressler, 2000). Thus, preventive strategies are of great importance.

The following sections present some of the evidence collected *in vitro*, in animals, and in humans suggesting an association between zeaxanthin, lutein and AMD. First, however, the mechanistic basis of this putative efficacy will be discussed.

3.4 Zeaxanthin and lutein in risk reduction of AMD

3.4.1 The mechanistic basis

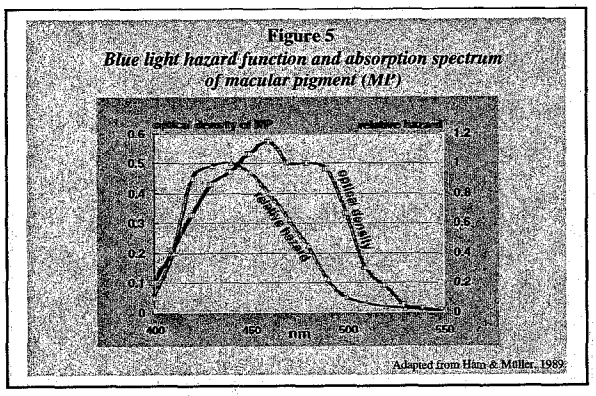


Figure 5
Blue light hazard function and absorption spectrum of macular pigment (MP)

Zeaxanthin and lutein appear yellow because they absorb blue light (blue being the complementary color of yellow). On the other hand, blue light can damage the retina (Ham & Müller, 1989; Gottsch et al., 1990), and this property of carotenoids is one basis of their physiological action in the retina. The relationship between the wavelength of blue light and its potential to induce damage in the retina is expressed by the "blue light hazard function" (Ham & Müller, 1989). This function has a maximum at around 450 nm, near the peak wavelength at which lutein and zeaxanthin absorb light. (Figure 5). Thus, these carotenoids can absorb blue light before it can initiate damaging reactions in the photoreceptors. Their location in Henle's fiber layer (Figure 1), just in front of the photoreceptors, is appropriate to their filter action and also explains the classical function of macular yellow pigment, namely the attenuation of chromatic aberration.

Blue light filtration by the macular pigment is probably of particular importance in youth until an age of between 30 and 40 years, when the lens is virtually clear. During the normal aging process, however, the lens turns yellow (Hockwin et al., 1984) producing an age-related reduction of its blue light transmission. Therefore, in later life, the antioxidant properties of zeaxanthin and lutein, described in the next section, may become even more important because the antioxidant system of the body deteriorates with age (Castorina et al., 1992).

3.4.2 Anti-oxidant properties of zeaxanthin

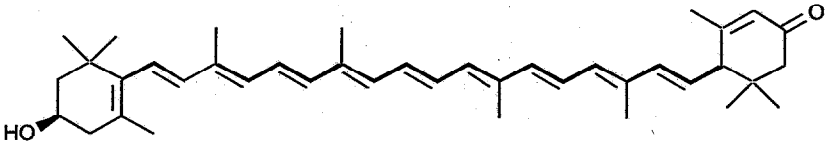
The screening effect described above attenuates blue light and thus indirectly limits the photochemical generation of reactive oxygen species mediated via endogenous or exogenous photosensitizers. However, carotenoids in general and zeaxanthin and lutein in particular also have intrinsic properties that directly quench these potentially damaging reactive entities (di Mascio et al., 1989; Conn et al., 1991). This quenching capability of carotenoids depends in part on the number of conjugated double bonds. As can be seen from Table 1, lutein has 10 conjugated double bonds, while 3R,3'R-zeaxanthin contains 11 such bonds. Therefore, at least in *in vitro* systems, 3R,3'R-zeaxanthin has a higher quenching capability for reactive species than lutein as can be seen in the Table 2 (Schalch et al. 1999).

Additional *in vitro* experiments (Mortensen et al, 1997) indicate that zeaxanthin is also more efficient than lutein in neutralizing some (e.g. the nitrogen dioxide radical (NO_2•) or the 2-mercaptoethanol thiyl radical ($HO(CH_2)_2S$•) but not all radical species (e.g. the glutathione thiyl radical (GS•).

Table 2		
Carotenoid	**Tocopheryl radical cation[a]**	**Singlet oxygen[b]**
3R,3'R-zeaxanthin	**26.4**	**12.6**
Lycopene	13.5	16.8
ß-carotene	10.2	13.5
Canthaxanthin	8.8	13.2
Lutein	**5.3**	**6.6**
2nd order rate constants $M^{-1}s^{-1}$; [a]: in hexane, [b]: in benzene		

However, in order to directly exert their quenching abilities, the carotenoids must be relatively close to the location where those entities are generated. Photoreceptors are most important in this respect since they have a high concentration of polyunsaturated fatty acids, in particular of docosahexanoic acid, that are easily oxidized in the simultaneous presence of light and oxygen. If zeaxanthin and lutein were exclusively localized in the photoreceptor axons, they would be too far away from the short-lived reactive molecules to quench them. However, two independent groups (Sommerburg et al., 1999; Rapp et al., 2000) have reported the occurrence of substantial quantities of zeaxanthin and lutein in photoreceptor outer segments, at the location where the probability of generation of reactive oxygen entities is greatest.

The hypothesis that the macular carotenoids are indeed involved in antioxidant reactions is supported by the identification of oxo-lutein (formula (1) below) by Khachik et al. (1997) in 58 pairs of postmortem human retinas and one pair of monkey retinas. While the amount of this molecule in the retina is substantial (up to 24% of total retinal lutein), it is not yet known whether its occurrence in the retina is specific or due to passive transport from the plasma.



Oxo-lutein (=(3R,6'R)-3-hydroxy-β,ε-carotene-3'-one) (1)

To summarize, the preponderance of 3R,3'R and 3R,3'S-zeaxanthin in the macular center, where the incident light is most intense is logical: These molecules have a higher quenching capability compared to lutein, that is relevant, because the likelihood of formation of reactive species is greatest at the center of the retina. The putative metabolic conversion of lutein into meso-zeaxanthin described previously would further support this view.

3.5 The animal *in vivo* evidence

Animal studies can be used to test whether mechanistic ideas developed on the basis of *in-vitro* experiments are clinically relevant. The problem regarding AMD is that only one good animal model exists to also allow the evaluation of the effect of carotenoids in the context of this disease. Primates qualify for such a model, because only they have a macula lutea and they are reported to

develop drusen and age-related macular changes similar to that in human AMD (Monaco & Wormington, 1990; Hope et al., 1992). However, they have not been widely utilized as model animals.

3.5.1 Studies in rats

Liu et al. (1995) reported probably the first animal study that tried to evaluate the potential protective effect of 3R,3'R-zeaxanthin on retinal light damage in the rat. They fed 1 g/animal/day of Gou Qi Zi berries, to 24 SD rats starting 4 days before a 24 hour exposure to intense fluorescence light (250 ft candles) and continuing for two days thereafter. At 3, 6 or 14 days after light exposure, damage to the retina was assessed by histology and compared to a control group. In the control group, rods and cones were severely injured. The number of nuclei in the ONL (outer nuclear layer) was significantly decreased and degeneration and necrosis of the retinal pigment epithelium was noted. In the animals that had received the 3R,3'R-zeaxanthin containing berries, however, rods and cones appeared normal, with only the number of photoreceptors being slightly decreased. This study has several weaknesses, and could not exclude with certainty whether the observed effects may have been caused by some other ingredients of the berries. Furthermore, the albino rat is not an ideal animal to study carotenoids and is ophthalmologically very different from humans. However, the effects could be caused by 3R,3'R-zeaxanthin and would be consistent with the hypotheses presented in the preceding sections as well as with the results of the experiments in quails using pure 3R,3'R-zeaxanthin described in the next section.

3.5.2 Studies in quails

Although quails lack a macula lutea, their cone-rich retina has characteristics similar to the human retina in that it accumulates carotenoids (Bowmaker et al., 1993) and can form drusen (Fite et al., 1994). Dorey et al.(1997) presented evidence supporting the idea that in this animal model, 3R,3'R-zeaxanthin has a preventive potential in regard of light damage. They fed carotenoid free diets that were specifically supplemented with 0.1, 0.3, 0.6, 5 or 50 mg/kg pure 3R,3'R-zeaxanthin (from *Flavobacter*) for three months to Japanese quails (*Cuturnix cuturnix japonica*). The animals were then exposed to intermittent white light (3200 lux) for 28 hours in order to induce general photic damage to the retina. After 14 hours in the dark, the eyes were excised for HPLC determination of 3R,3'R-zeaxanthin in the retina and measurement of the extent of apoptosis, including TUNEL (TdT-mediated dUTP nick end-labeling) staining. The number of light-induced apoptotic rod and cone photoreceptor cells was drastically reduced in treated animals. Furthermore, the retinas containing more 3R,3'R-zeaxanthin (as assessed by HPLC) seemed to be better protected than those with less.

In an extension of the study, the authors also investigated quails that were raised in dim light and supplemented for one year with carotenoid free diets containing 0.5 or 40 mg/kg pure 3R,3'R-zeaxanthin. A number of morphological parameters were assessed and correlated with the concentration of 3R,3'R-zeaxanthin in serum and retina. The authors concluded that 3R,3'R-zeaxanthin supported the aging retina (Dorey et al., 1998).

These experiments can be considered to be the first pre-clinical demonstration of the efficacy of pure 3R,3'R-zeaxanthin in the eye.

3.5.3 Studies in other non-primate animals

Another animal that may prove useful for the study of zeaxanthin and lutein in the eye is the frog. Recently, meso-zeaxanthin was detected in the retina but not in the liver of the frog (*Rana pipiens*) (Khachik et al., 2000), indicating that the metabolism of macular carotenoids in this aquatic animal may be similar to that in humans.

3.5.4 Studies in primates

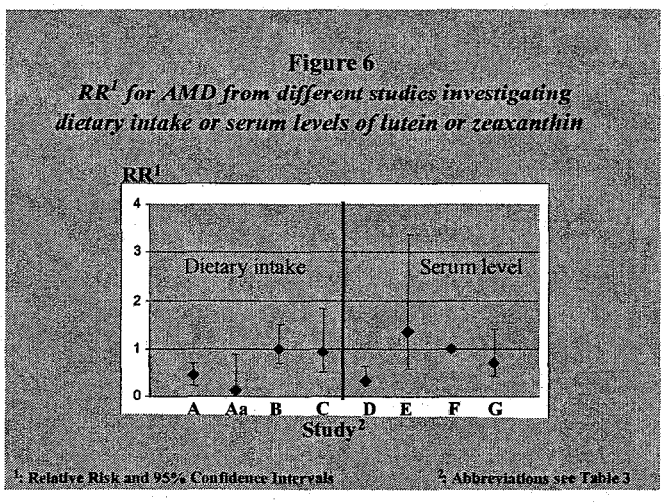
The best animals to investigate questions related to carotenoids in the macula are primates, because they have a macula lutea very similar to those of humans. One of the first questions was if the yellow spot is indeed of nutritional origin and if it disappeared when a carotenoid depleted diet would be ingested. For this purpose, macaque monkeys were fed a carotenoid deficient diet for 2 to 6 years (Malinow et al., 1980). This led to disappearance of plasma carotenoids and more gradual disappearance of the yellow pigmentation of the retina. Furthermore, fluorescence angiography documented various defects of the retina, including window defects. In comparison with normally fed monkeys of the same age, the depleted monkeys showed also more drusen that in humans is one of the hallmarks of AMD.

3.6 Investigations in humans

3.6.1 Observational studies

Haegerstrom-Portnoy et al. (1988) reported that the age-induced decline of retinal sensitivity of the blue-sensitive cones is slower in areas where macular yellow pigment is present. Later, and consistent with this observation, Hammond et al. (1998) found an accelerated decrease in short-wavelength (blue) cone sensitivity in older individuals with lower macular pigment concentrations. Furthermore, subjects with high levels of macular yellow pigment had a retinal sensitivity similar to young subjects, as if the presence of macular pigment had conserved their retinal sensitivity. The authors felt that these results support the hypothesis that carotenoids reduce the risk for AMD, as the deterioration in retinal sensitivity and acceleration of blue cone loss that accompany aging are known to precede the clinical manifestation of AMD and other macular diseases (Sunness et al., 1989).

Figure 6
RR[1] for AMD from different studies investigating dietary intake or serum levels of lutein or zeaxanthin



[1]: Relative Risk and 95% Confidence Intervals
[2]: Abbreviations see Table 3

A number of toxic (such as that caused by the photosensitizing drug chloroquine (Bernstein & Ginsberg, 1964)) and degenerative changes in the retina show an annular pattern, called Bull's-eye maculopathy. In these conditions, a circular ring of structural change, surrounding and mostly

sparing the macula, can be seen. The fovea itself does not seem to be affected. In a study involving 95 subjects, Weiter et al. (1988) found that the area spared from the degenerative changes corresponds closely to the area with the highest concentration of macular pigment and at the same time with the highest concentration of zeaxanthin and meso-zeaxanthin. The authors concluded that the presence of the macular pigment may have provided some protection and therefore spared the area covered by the pigment from the degenerative changes.

A more direct association of carotenoids with AMD is provided by the following study. In an HPLC study on postmortem eyes from 12 normal subjects and 12 subjects with AMD, Landrum et al. (1996) found average lutein and zeaxanthin levels to be approximately 30% lower in the AMD retinas than in the normal retinas. This difference was greatest in the central disc, tending to decrease in the medial and especially the outer annulus (see section 3.2). Therefore, it is possible that AMD at least partly contributes to the preferential loss of lutein and zeaxanthin in the macula.

Table 3

Study*	Total number of subjects (comments)	Reference
A	n = 876 (spinach 2-4/week)	Seddon et al., 1994
Aa	n = 876 (spinach daily)	Seddon et al., 1994
B	n = 1968	Mares-Perlmann et al., 1996
C	n = 1036	EDDC, 1993
D	n = 130	Sanders et al., 1993
E	n = 132	Alpers et al., 1995
F	n = 334	Mares-Perlmann et al., 1995
G	n = 1709	VandenLangenberg et al., 1998

*: Same abbreviations as in Figure 5.

With this reasoning, they have recently (Bone et al., 1999) refined their analyses and now investigated the retinas of a total of 56 AMD cases and 45 controls. The control subjects were divided into quintiles of carotenoid concentration in the outer annulus — which normally is unaffected by AMD — and the number of AMD cases in each quintile was determined so as to calculate the relative risk ratio. Comparison of individual relative risk ratios revealed a substantial and statistically significant lower of risk in the highest quintile of carotenoid concentration in the outer annulus. While it is still possible that decreased carotenoid levels even in the outer annulus could be a result rather than the cause of AMD, these data are consistent with some of the epidemiological studies to be discussed in the next section.

3.6.2 Epidemiological studies

The next pieces in the puzzle of an emerging role of carotenoids in risk reduction of AMD are epidemiological studies. Such studies generally determine the risk for AMD in specifically selected populations relative to a control group.

The epidemiological study that is probably most relevant in this respect is a case control study that compared plasma carotenoid concentrations of 356 subjects with neovascular AMD with 520 control subjects. This study found a statistically significant inverse relationship between plasma levels of lutein and zeaxanthin and the risk for neovascular AMD (EDDC, 1993), i.e. higher concentrations with a lower AMD risk. These results are consistent with another paper from the same study showing a lower risk for AMD in subjects with a higher dietary intake of lutein and zeaxanthin (Seddon et al., 1994). When the authors tested correlations with specific foods, they found that subjects who consumed a medium-sized portion of spinach (approximately 75 g of spinach) two to four times a week were found to have a statistically significant 46% reduction in the risk for AMD (study A in Figure 6) as compared with a control group, while subjects who ate spinach daily had their risk reduced by over 80% (study Aa in Figure 6). The consistent findings of this study between plasma, total dietary intake and single food item data are cited as key supporting evidence for the relationship between macular carotenoids and AMD.

However, the epidemiological relation of lutein and zeaxanthin to the risk of AMD is not yet totally clear. The Beaver Dam Study (Mares-Perlman et al., 1995 & 1996) is an epidemiological study that examined a largely Caucasian community in south-central Wisconsin, comparing 167 subjects with retinal pigment epithelial abnormalities, soft drusen, or exudative AMD with an equal number of normal control subjects. Individual plasma concentrations of lutein and zeaxanthin were found to be slightly, though not statistically significantly, lower in subjects with exudative macular degeneration.

In Figure 6, results from the few epidemiological studies that have evaluated the relative risk for AMD in respect to lutein or zeaxanthin plasma or intake levels are compared. While the epidemiological evidence may appear to be conflicting, it has to be appreciated that AMD is multi-factorial and is a difficult disease to study. AMD has a long time-course and etiologically may be initiated very early in life. Plasma levels and dietary intake of lutein and zeaxanthin are considered to be good parameters for delineating the influence of present nutrition on the development and progression of AMD. Thus, the information they provide relates to current dietary intake, whereas long-term dietary history may be of particular significance, especially as AMD is a disease of senescence.

3.6.3 Intervention trials

While epidemiological studies cannot provide definite proof of the efficacy of lutein and zeaxanthin in AMD, such studies can provide evidence of possible relationships but cannot determine whether an effect is causal. The situation is different with intervention studies in which agents are administered on a double-masked, placebo-controlled, and randomized basis and results are evaluated using predefined efficacy parameters. Only supplementation studies with lutein and zeaxanthin are likely to provide a definite answer as to an effect of lutein and zeaxanthin on AMD (Seddon & Hennekens, 1994). Nevertheless, the specific time-course and nature of this disease make the design of such trials very difficult.

To date, no well-controlled intervention trials with lutein or zeaxanthin have been published. One reason for this is that until recently lutein and zeaxanthin supplements were not widely available for human consumption.

Neither of these carotenoids is being used in the ongoing clinical study within the Age-Related Eye Disease Study (AREDS Research Group, 1999). This study was started by the National Eye Institute in 1992, before lutein became readily available. Its epidemiological part investigates the natural history of AMD and cataract, while its clinical part evaluates the effect of high-dose vitamin (including β-carotene) and mineral supplements on AMD and the effect of high-dose vitamin supplements on cataract.

3.7 Other possible ophthalmological effects

The results of two recent studies may provide preliminary support for an early hypothesis that macular pigment improves visual performance by absorbing blue light and attenuating chromatic aberration, effects which may influence visual function parameters such as visual acuity. An open-label study with self-selected subjects by Zorge et al. (1999) reported significantly improved visual function (including visual acuity) in 20 patients with congenital retinal degenerations who chose to increase their intake of lutein. In a case report series, Richer et al. (1999) reported visual function testing in 13 subjects with various ophthalmological diseases of the macula many of whom had been advised to increase their consumption of spinach, a rich source of lutein (see Table 6). They reported that virtually all subjects were found to have improvements in a number of visual function tests including contrast sensitivity. Additional, well-controlled clinical trials are needed to confirm these preliminary findings.

3.8 Modulation of macular pigment density and plasma concentrations of zeaxanthin and lutein

One important question relates to the specific bioavailability of the test substance at the target organ, i.e. Is it possible to increase the amount of lutein and/or zeaxanthin in the macula by means of dietary manipulation or by supplementation with the pure compounds? This question has been investigated in various studies.

3.9 Modulation by diet

The response of macular pigment density to dietary administration of lutein and zeaxanthin was investigated by Hammond et al. (1997a). For up to 15 weeks, volunteers were given a diet that was rich in spinach (providing 10.8 mg lutein and 0.3 mg zeaxanthin per day) and/or corn (providing 0.4 mg lutein and 0.3 mg zeaxanthin per day). The carotenoid concentrations in plasma and optical densities in the retina were measured. One volunteer showed no increase in the levels of the carotenoids neither in the plasma nor in the macula. Two volunteers were found to have increased concentrations in the plasma, but not in the macula. The remaining nine volunteers, however, showed increased concentrations of lutein and zeaxanthin both in the plasma (up to about 33% from baseline) and in the macula (up to about 19%). In one of the two subjects who received the corn diet only, plasma zeaxanthin increased by 70% and macular pigment by 25% respectively, while the other did not respond at all. It was therefore concluded that although the response to

dietary lutein and zeaxanthin ingestion varies considerably, the amount of carotenoids in the macula can be increased by dietary modification.

A recent paper (Johnson et al., 2000) once again investigated the effect on macular pigment density of ingesting daily portions of spinach and corn containing a total of 11.2 mg lutein and 0.6 mg zeaxanthin in addition to the usual diet. After four weeks, plasma lutein had increased almost twofold, while plasma zeaxanthin had increased by only a small, though statistically significant, amount. The latter finding is not surprising given the relatively small amount of zeaxanthin ingested. To put this into perspective in comparison with lutein, the authors report the mean peak serum concentration per amount of carotenoid ingested as 20 nM/μmol for lutein and 24 nM/μmol for zeaxanthin, indicating that the bioavailability of these two carotenoids from food is not likely to be very different. Concomitantly with the increase in plasma lutein and zeaxanthin, there was also a small but statistically significant increase in macular pigment as measured by heterochromatic flicker photometry. While plasma levels of lutein had returned to baseline levels two months after cessation of the additional dietary lutein and zeaxanthin intake, macular pigment density was still significantly higher than at baseline. The conclusion of similar availability of lutein and zeaxanthin from food sources is also supported by another recent investigation. During this work, different diets were supplemented with egg yolks containing known amounts of lutein and zeaxanthin and were given to volunteers for 4.5 weeks (Handelman et al., 1999). The plasma concentration increments normalized to the amount of carotenoid ingested were almost identical for lutein and zeaxanthin in the group ingesting beef tallow supplemented with egg yolk.

3.10 Modulation by supplementation

The plasma response of monkeys to feeding with synthetic zeaxanthin formulated into a beadlet formulation was studied in three animals. The plasma concentration of zeaxanthin increased dose dependently reaching a level of around 500 nM approximately 3 weeks after begin of supplementation with a daily dose of 2.5 mg (equivalent to 2.8 mg/kg/day) (Snodderly et al., 1997). In this study, macular pigment of the supplemented animals was not assessed.

Khachik et al. (1995) purified lutein from marigold flowers and zeaxanthin from the berry *Lycium barabarum*, Gou Zi Qi (Table 6), and administered suspensions in olive oil to three volunteers. Daily doses of 10 mg were given for 18 (lutein) or 21 (zeaxanthin) days. Analyses by HPLC showed that the serum levels of both carotenoids peaked after one week: lutein at 1.4 μmol/L, zeaxanthin considerably lower at 0.1 μmol/L. No explanation was offered for this large difference. Another study measured uptake of lutein into the plasma after supplementation with capsules containing an extract of marigold flowers in corn oil (Olmedilla et al., 1997b). For three months, nine volunteers were supplemented with 15 mg lutein. After one month, independently of initial lutein levels (mean 0.3 μmol/L), plasma concentrations increased three to fivefold.

Landrum et al. (1997) measured plasma carotenoids concurrently with macular pigment density. This was a supplementation study with two subjects receiving 30 mg lutein (as a marigold lutein ester extract suspended in canola oil) daily for 140 days. Serum lutein levels rapidly increased tenfold from 0.2-0.3 μmol/L in the first week and maintained that level for the remainder of the study. Macular pigment density as estimated by heterochromatic flicker photometry showed a slower response than the serum levels, starting to increase after approximately 20 days. In one

subject, macular pigment density had increased by an average of 41% and 37% in the right and left eye respectively, and by 21% bilaterally in the other subject at the end of the supplementation period, after which, however, it appeared to continue to increase for another month. The authors concluded that increase in macular yellow pigmentation appears to be a slow process with considerable inter-individual variation. The same authors also supplemented a small number of volunteers over a duration of 4 months with 30 mg per day of pure 3R,3'R-zeaxanthin (from *Flavobacter*) that was formulated into gelatin/starch beadlets (Bone et al., 1998). Following 10 to 20 day of supplementation, plasma levels had reached a plateau at a concentration of approximately 0.5 μM, almost six folds higher than at baseline. Approximately 40 days after starting the supplementation, macular pigment densities had also started to increase.

Though there appears to be considerable variability, these results demonstrate that macular pigment can indeed be altered by supplementation or by diet. The level of the macular pigment in the eye has been shown to be very stable over time (Bone et al., 1988 and Hammond et al., 1997b). In this respect, Hammond et al. (1997a) reported on a subject whose macular pigment optical density was very stable over five years, yet increased by 50% after only 14 weeks of a test diet rich in lutein and zeaxanthin and remained elevated for nine months after the diet was discontinued.

3.11 Recommended Intake

The Eye Disease Case-Control Study (EDCC, 1993; Seddon et al, 1994) found that persons in the highest quintile of lutein + zeaxanthin intake had a significantly lower relative risk of neovascular AMD (age-related macular degeneration) compared to controls. Median intake in this quintile was 5.757 mg/d (normalized for calorie intake). In a different age-related eye disease that may also be associated with intake of lutein and zeaxanthin, the Beaver Dam Follow-up Eye Study (Lyle et al, 1999), Nurse's Health Study (Chasen-Taber et al, 1999) and Health Professionals Follow-up Study (Brown et al, 1999) all found a statistically significant trend toward lower risk of cataract extraction among persons with higher intakes of lutein + zeaxanthin. The difference in relative cataract risk between the highest and lowest quintiles of intake only reached statistical significance in Lyle et al (1999). The range of intakes in Chasen-Taber et al (1999) and Brown et al (1999) encompassed 6 mg/d, and relative risk was apparently (though not significantly) lower around and above this level. From these studies, one might hypothesize that dietary intake near or above 6 mg/d has the potential to be associated with a relatively lower risk of age-related eye disease. Any more definitive recommendations need to be based on intervention and efficacy trials.

Recently, Mohamedshah et al. (1999) presented an analysis of average dietary lutein and zeaxanthin intake using the extensive ENVIRON food intake database (ENVIRON, 1999), the CSFII 1994-96 food intake survey and the 1998 USDA Carotenoid Composition of Foods database. As shown in Table 4, the estimated dietary intake ratio of lutein to zeaxanthin from the whole diet varies roughly between 4:1 and 6:1 across age groups, with an average of 5:1.

Table 4: Daily intake of lutein and zeaxanthin by age group calculated from CSFII 1994-96 intake data and the 1998 USDA Carotenoid Database (Mohamedshah et al, 1999).

Age Group	Lutein (μg/d)	3R, 3'R-Zeaxanthin (μg/d)	Lutein : Zeaxanthin Ratio
20-29	745	178	4.2 : 1
30-39	896	174	5.1 : 1
40-49	920	187	4.9 : 1
50-59	1053	182	5.8 : 1
60-69	1056	170	6.2 : 1
70+	990	170	5.8 : 1

Based on the available data, it is reasonable to assume that the ratio of lutein to zeaxanthin in the diets examined by the epidemiological trials (Table 5) would be similar in aggregate to that reported by ENVIRON (although absolute intake estimates among population subgroups could vary widely).

Table 5: Epidemiological studies reporting risk odds ratios for cataracts or AMD by quintile of lutein + zeaxanthin intake.

		Odds Ratios of AMD Risk Across Quintiles of Lutein + Zeaxanthin Intake#					
Study	**Outcome**	**1st**	**2nd**	**3rd**	**4th**	**5th**	**Trend**
Brown et al, 1999	Cataracts	*1300* **1.00**	*2279* **1.00** (0.81, 1.23)	*3182* **0.98** (0.79, 1.20)	*4342* **0.83** (0.67, 1.04)	*6871* **0.81** (0.65, 1.01)	0.03
Chasen-Taber et al, 1999	Cataracts	*1172* **1.00**	*2064* **1.01** (0.86, 1.19)	*2817* **0.95** (0.80, 1.11)	*6047* **0.81** (0.69, 0.96)	*11685* **0.88** (0.75, 1.03)	0.04
Lyle et al, 1999	Cataracts	*596** **1.0**	*918** **0.9** (0.6, 1.6)	*1200** **0.9** (0.6, 1.7)	*1568** **0.7** (0.4, 1.2)	*2490** **0.5** (0.3, 0.8)	0.002
Mares-Perlman et al, 1996	Early AMD[1]	*310** **1.0**				*1728** **1.0** (0.7, 1.5)	0.75
Seddon et al, 1994	"Wet" [2] AMD	*561* **1.00**	*1211* **1.14** (0.7, 1.8)	*1708* **0.84** (0.5, 1.3)	*2487* **0.77** (0.5, 1.2)	*5757* **0.43** (0.2, 0.7)	<0.001

Intakes (in μg/d) are listed in *italics*, risk odds ratios are listed in bold and 95% C.I. are listed in parentheses
Daily intake arbitrarily set at 2000 kcal/d (values originally reported as ug/1000 kcal)
[1] AMD: Age-related Macular Degeneration
[2] "Wet" AMD: neovascular form of AMD

With a putative recommended intake of 6 mg/d (or higher) lutein + zeaxanthin based on observational studies (Table 5) and an estimated average dietary intake ratio of lutein : zeaxanthin around 5:1 (Table 4), by extrapolation, the recommended dietary intakes should be approximately 5 mg/d lutein and approximately 1 mg/d zeaxanthin. The actual lutein + zeaxanthin efficacious intake could be higher since no plateau effect was observed in Seddon et

al (1994), but there are inadequate dose-response data to serve as a basis for a higher recommended level. Such a recommendation is considerably higher than average observed intakes in Mohamedshah et al (1999), which were derived from 24-hour dietary recall data (Table 4). But, the recommendation is not so unusual when compared to the highest quintiles of intake (i.e. 20% of the participants) reported by Brown et al (1999), Chasen-Taber et al (1999) and Seddon et al (1994) which, at an assumed lutein : zeaxanthin ratio of 5:1, could provide 0.98, 1.67 and 0.82 mg zeaxanthin per day, respectively, based on food-frequency questionnaire data.

Therefore, based on dietary intake, a recommended intake of approximately 1 mg 3R, 3'R zeaxanthin per day is consistent both with studies of diet and AMD risk and with intakes observed among a significant number of people surveyed in several epidemiological trials.

3.12 Summary

In the center of the retina where visual acuity is highest, a yellow spot called the macula lutea is visible. The yellow color is due to the presence of the nutritional carotenoids lutein and zeaxanthin, which are specifically accumulated there to a greater extent than in any other tissue. In the center of the retina, the lutein-to-zeaxanthin ratio is much lower than that found in the plasma. This selective accumulation may be physiologically significant based on filtration of potentially damaging blue light, quenching of photochemically-induced reactive oxygen species, attenuation of chromatic aberration, and inhibition of apoptosis. It is believed that via these mechanisms, lutein and zeaxanthin may contribute to a reduced risk of age-related macular degeneration (AMD), the leading cause of irreversible loss of vision in the US. Epidemiological studies indicate that elevated dietary intake or blood concentrations of lutein and zeaxanthin are correlated with a reduction in the risk for this disease. Furthermore, intake of these carotenoids can specifically increase their levels in the macula.

Based on dietary intake, a recommended intake of approximately 1 mg 3R, 3'R zeaxanthin per day is consistent both with studies of diet and AMD risk and with intakes observed among a significant number of people surveyed in several epidemiological trials.

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Chapter 4: Nutritional aspects – Review of Available Data on Existing Human Exposure from Diet

Of the approximately 600 known carotenoid molecules, only about 50 carotenoids are found in the diet, particularly in yellow/orange/red fruits and dark green leafy vegetables (Kull et al., 1995). About 13 carotenoids have been identified in human plasma (Khachik et al., 1997) with five (α-, and β-carotene, β-cryptoxanthin, lycopene and lutein) occurring there in substantial quantities and with zeaxanthin as a comparatively minor constituent. However, in the macula, only two diet-derived carotenoids are present, 3R,3'R-zeaxanthin and lutein. These molecules are called the macular carotenoids. Given their presence in the retina, a valid question is whether they are involved in the visual cycle as a result of pro-vitamin A activity. No substantial vitamin A activity of macular carotenoids has been detected (Weiser et al., 1993), indicating that they can not provide retinol to the retina and must act in a different mechanistic way.

Table 6: Lutein and zeaxanthin content and ratios of common foods. Shaded bars indicate foods with a higher concentration of zeaxanthin than lutein. (Source: USDA Carotenoid Database, 1998, unless otherwise noted: [1] Lam & But, 1999; [2] Müller, 1997)

Food	Form	Lutein (μg/100g)	3R, 3'R-Zeaxanthin (μg/100g)	Lutein: Zeaxanthin Ratio
Beans	snap, green, canned, regular pack	616	44	14 : 1
Broccoli	Cooked	2203	23	96 : 1
Carrots	baby, raw	335	23	15 : 1
Celery	Cooked	242	8	31 : 1
Celery	Raw	229	3	77 : 1
Collards	Cooked	7825	266	30 : 1
Corn	sweet, yellow, canned, whole kernel	356	528	0.68 : 1
Corn	Cornmeal, degermed, enriched, yellow	898	457	2 : 1
Egg	whole, raw, fresh	32	23	2 : 1
"Gou Qi Zi" berry[1]	Raw	-	2340	-
Kale	Cooked	15625	173	91 : 1
Lettuce	cos or romaine, raw	2448	187	14 : 1
Lettuce	iceberg (includes crisphead types), raw	282	70	5 : 1
Orange	juice, frozen concentrate	58	80	0.73 : 1
Orange	raw, all commercial varieties	113	74	2 : 1
Pea	green, canned, regular pack	1292	58	23 : 1
Peach	canned, heavy syrup	14	19	0.74 : 1
Peach	Raw	51	6	9 : 1
Pepper, red[2]	Raw	0.00	2.20	-
Persimmons	japanese, raw	346	488	0.71 : 1
Spinach	Raw	11607	331	36 : 1
Spinach	Cooked	6864	179	39 : 1
Tangerine	Raw	131	112	2 : 1
Turnip greens	Cooked	8173	267	31 : 1

For humans, who cannot synthesize carotenoids, food is the exclusive source of the macular carotenoids. The zeaxanthin that occurs in vegetables and fruits is exclusively the 3R,3'R-zeaxanthin and none of its optical stereoisomers, neither 3R,3'S nor 3S,3'S-zeaxanthin, naturally

occur in plants. As can be seen in Table 6, many vegetables and fruits contain markedly more lutein than 3R,3'R-zeaxanthin (e.g. kale, spinach, broccoli).

In human plasma, the concentration of lutein is also generally much higher than that of 3R,3'R-zeaxanthin (Table 1). More 3R,3'R-zeaxanthin than lutein is found in some plant products: corn, oranges (frozen juice), peaches (processed), persimmons, red or orange bell peppers and the small red berry *Lycium barbarum*, "Gou Qi Zi" (Table 6). This berry has been commonly used for home-cooking in China as well as traditional Chinese herbal medicine, where it is used to improve visual acuity. Historically, this may have been the first ophthalmological use of 3R,3'R-zeaxanthin. According to Lam and But (1999), the mean concentration of zeaxanthin in commercially available berries was 2340 μg/ 100 g; it varied from 1150 to 4330 mcg/100 gm (mean values of 5 analyses/ for each berry lot from six different sources) .

4.1 Introduction

The purpose of this section is to provide a review and discuss the available literature on daily consumption of zeaxanthin from food sources. Other than dietary supplementation, food is the exclusive source of zeaxanthin in humans. Humans do not have the ability to synthesize zeaxanthin *de novo*. The zeaxanthin that occurs in vegetables and fruits is the 3R,3'R-zeaxanthin stereoisomer.

The standard HPLC method for analyzing carotenoids in food does not distinguish between lutein and zeaxanthin (Khachik et al. 1991). As a result, analytical data are generally reported as the sum of lutein + zeaxanthin. Eight studies reporting lutein + zeaxanthin intake in US populations are reviewed here (see below). In 1998, the USDA published a food composition database that listed the zeaxanthin content of 16 individual foods analyzed by different methodology. Two studies (also reviewed here) have used this database to calculate zeaxanthin intake in US populations (Mohamedshah et al. 1999; Slattery et al. 2000). A method of estimating zeaxanthin intake from lutein + zeaxanthin data by using a dietary ratio of 5:1 lutein to zeaxanthin is described in Section 4.2.1.

4.2 Review of available studies on Human Exposure (Intake)

Three dietary intake assessment methods have been used to assess intake of carotenoids (including zeaxanthin) from food – diet history method, the food frequency questionnaire (FFQ) and 24-hour recall data methods. The available data collected with all three methods are presented below.

4.2.1 24-hour Dietary Recall Methodology in a Nationally Representative Sample

Mohamedshah et al. (1999) studied 9,323 individuals who participated in the 1994-1996 USDA Continuing Survey of Food Intakes by Individuals (CSFII). The CSFII study was designed using sampling and statistical procedures that allow prediction of dietary intakes across a representative sample of the US population. Dietary intake data were collected by asking

participants to provide 24-hour dietary recall data on two separate days. Lutein and zeaxanthin intake were calculated separately using the 1998 USDA-NCC Carotenoid Database supplemented by unpublished data from the USDA Nutrient Data Laboratory (G. Beecher, personal communication). Results are shown in Table 7 and Table 8.

Table 7: Average Intake of Zeaxanthin by Age Group and Gender (Mohamedshah et al., 1999)

Age	Zeaxanthin (µg/day)	
	Male	Female
20 – 29	191	163
30 – 39	190	159
40 – 49	213	160
50 –59	210	158
60 – 69	193	151
70 and above	184	160

Table 8: Average Intake of Lutein and Zeaxanthin by Age Group (Mohamedshah et al., 1999).

Age Group	Lutein (µg/d)	3R, 3'R-Zeaxanthin (µg/d)	Lutein : Zeaxanthin Ratio
20-29	745	178	4.2 : 1
30-39	896	174	5.1 : 1
40-49	920	187	4.9 : 1
50-59	1053	182	5.8 : 1
60-69	1056	170	6.2 : 1
70+	990	170	5.8 : 1

Other than the study by Slattery et al. (2000) (see section 4.2.2), all other US dietary intake studies in this review have reported lutein + zeaxanthin intake in combination rather than individually. In order to estimate zeaxanthin intake from studies reporting lutein + zeaxanthin intake, the ratio of lutein : zeaxanthin in the US diet was calculated using the data from Mohamedshah et al. (1999). Table 8 shows that the ratio of lutein : zeaxanthin in whole diets ranged from 4.2:1 to 6.2:1 depending on the age range studied.

Therefore, for purposes of estimating zeaxanthin intake in this document, a ratio of 5:1 (lutein: zeaxanthin) will be employed.

4.2.2 Diet History Methodology in a non-Representative Sample

Slattery et al. (2000) studied 1993 colon cancer cases and 2410 healthy controls drawn from the Kaiser Permanente Medical Care Program in Northern California, Utah and the Twin Cites of Minnesota. Dietary intake data were collected using a diet history questionnaire and carotenoid

intake was calculated using the 1998 USDA-NCC carotenoid database. Lutein and zeaxanthin intake were calculated separately. Among colon cancer cases, men consumed 161 ± 115 μg and women consumed 169 ± 163 μg zeaxanthin per day while, among healthy controls, men consumed 164 ± 122 μg and women consumed 155 ± 107 μg.

4.2.3 Food Frequency Questionnaire Methodology in a Nationally-Representative Sample

Nebeling et al. (1997) studied 8,161 participants in the 1987 National Health Interview Survey (NHIS) and 8,341 participants in the 1992 NHIS. While the NHIS is a nationally-representative sample of resident, non-institutionalized US citizens, this study only included data collected from Whites and African-Americans (blacks). Dietary intake data were collected using a FFQ and lutein + zeaxanthin intake was calculated using data from Chug-Ahuja et al. (1993). Mean (± standard error) lutein + zeaxanthin intake among men was 2.15 ± 0.05 mg per day in 1987 and 1992. Among women, mean (± standard error) intake was 2.21 ± 0.04 mg in 1987 and 1.86 ± 0.03 mg in 1992.

4.2.4 Food Frequency Questionnaire Methodology in non-Representative Samples

Seddon et al. (1994) studied 356 neovascular macular degeneration cases and 520 healthy controls in the Eye Disease Case-Control Study. Dietary intake data were collected using a FFQ and lutein + zeaxanthin intake was calculated using data from Mangels et al. (1993). Median lutein + zeaxanthin intake in the middle (3^{rd}) quintile was 1708 μg per day.

Vandenlangenberg et al. (1996) studied 2,152 participants (ages 43-85) in the Nutritional Factors in Eye Disease Study in Beaver Dam, WI. Dietary intake data were collected using FFQ and lutein + zeaxanthin intake was calculated using data from Mangels et al. (1993. Mean lutein + zeaxanthin intake was 962 ± 642 μg per day. Median intake was 811 μg per day.

Yuan, et al (1998) studied 1204 renal cell carcinoma patients and 1204 healthy controls selected from the Los Angeles County Cancer Surveillance Program. Asian-Americans were excluded from this study population. Dietary intake data were collected using a FFQ and lutein + zeaxanthin intake was calculated using carotenoid composition databases from the Cancer Research Center of Hawaii (unpublished) and Mangels et al. (1993). The median (3^{rd}) quintile of lutein + zeaxanthin intake ranged from 1161 μg at the low cutoff to 1555 μg at the high cutoff.

Tucker et al. (1999) studied 230 men and 346 women (aged 67 – 93) participating in the Framingham Heart Study. Dietary intake was measured using FFQ and lutein + zeaxanthin intake was calculated using data from Mangels et al. (1993). Mean lutein + zeaxanthin was 2678 ± 1934 μg/day for men and 3087 ± 2380 μg/day for women. Median intake was 2298 μg for men and 2522 μg for women.

Brown et al. (1999) studied 36,644 men (ages 45-75) participating in the Health Professional's Follow-up Study. Dietary intake was measured using FFQ and lutein + zeaxanthin intake was

calculated using data from Tonucci et al. (1995) and Mangels et al. (1993). Median lutein + zeaxanthin intake in the middle (3rd) quintile was 3182 μg per day.

Chasen-Taber et al. (1999) studied 77,466 women (ages 45-71) participating in the Nurse's Health Study. Dietary intake was measured using FFQ and lutein + zeaxanthin intake was calculated using data from Tonucci et al. (1995) and Mangels et al. (1993). Median lutein + zeaxanthin intake in the middle (3rd) quintile was 2817 μg per day.

Cohen et al. (2000) studied 628 prostate cancer cases and 602 healthy male controls from the Seattle, WA, area. Dietary intake was measured using FFQ and lutein + zeaxanthin intake was calculated using the 1998 USDA-NCC carotenoid database. Mean daily intake of lutein + zeaxanthin was 1211 ± 796 μg in cases and 1260 ± 868 μg in controls.

4.2.5 Tabulated Food Frequency Questionnaire Data from non-Representative Samples

Seven studies using the FFQ method of determining zeaxanthin exposure have been reported in the literature. The results of these studies are tabulated in Table 9.

Table 9: Summary of Studies Reporting Lutein + Zeaxanthin Intake Using the FFQ Method

STUDY NAME	SAMPLE SIZE	ALL SUBJECTS (μg /day)	MALE (μg/day ± SD)	FEMALE (μg/day ± SD)	REFERENCE
Studies Reporting Median Values					
Eye Disease Case Control Study	876	1708			Seddon et al., 1994
Nutritional Factors in Eye Disease Study	2152	811			Vandenlangenberg et al., 1996
Los Angeles County Cancer Surveillance Program	2408	1161-1555 (low-high cutoff)			Yuan et al., 1998
Framingham Heart Study	576		2298	2522	Tucker et al., 1999
Health Professionals Follow-up Study	36644		3182		Brown et al., 1999
Nurse's Health Study	77466			2817	Chasan-Taber et al., 1999
Studies Reporting Mean Values (± SD)					
Nutritional Factors in Eye Disease Study	2152	962 ± 642			Vandenlangenberg et al., 1996
Seattle, WA area	628 cases	1211 ± 796			Cohen et al., 2000
Seattle, WA area	602 controls	1260 ± 868			Cohen et al., 2000
Framingham Heart Study	576		2678 ± 1934	3087 ± 2380	Tucker et al., 1999

4.3 Comparison and Validation of Methodologies

The methods used to calculate lutein + zeaxanthin intake in various populations have been validated or compared in the following studies:

Michaud et al. (1998) showed that FFQ data is reproducible. They provided FFQ on two occasions (one year apart) to 157 men from the Health Professional's Follow-Up Study and 197 women from the Nurse's Health Study (Table 10). Lutein + zeaxanthin intake was calculated using data from Chug-Ahuja et al. (1993) and Mangels et al. (1993).

Table 10: Lutein + Zeaxanthin Intake Calculated Using FFQ Given on Two Occasions One Year Apart (Michaud Et Al., 1998)

GENDER	LUTEIN+ZEAXANTHIN FFQ1 (µg/day)	LUTEIN+ZEAXANTHIN FFQ2 (µg/day)
Men	3784 ± 2382	3803 ± 2038
Women	4438 ± 3674	3984 ± 2690

VandenLangenberg et al. (1996) showed that different food composition databases can produce comparable lutein + zeaxanthin intake estimates. They studied 2,152 participants (ages 43-85) in the Nutritional Factors in Eye Disease Study in Beaver Dam, WI (Table 11). Dietary intake data were collected using FFQ and lutein + zeaxanthin intake was calculated using data from Mangels et al. (1993) or from an unpublished database accompanying the Block-NCI Health Habits and History Questionnaire (HHHQ). A composite of the USDA-NCI / HHHQ data was also reported.

Table 11: Lutein + Zeaxanthin Intake Calculated Using FFQ and Two Different Food Composition Databases (Vandenlangenberg Et Al., 1996)

Data Source	Mean ± SD(µg/day)
HHHQ	816 ± 622
USDA-NCI	962 ± 642
Composite	1089 ± 661

Katsouyanni et al. (1997) showed that carotenoid intake data collected using FFQ methodology produces higher intake estimates than data collected from the same people using 24-hour recall methodology. They studied 42 men and 38 women (ages 25-67) participating in the European Prospective Investigation into Cancer and Nutrition (EPIC) recruited from the Athens area of Greece. Dietary intake data was collected twice using FFQ or twelve times using 24-hour dietary recalls. Beta-carotene intake was calculated using data from a local food composition table (A. Trichopoulou, unpublished data). For men, ß-carotene intake was 3.6 ± 2.1 per day using 24-hour dietary recall, 6.6 ± 4.7 mg per day in the first FFQ and 7.6 ± 6.9 mg per day in the second FFQ. For women, the results were 2.8 ± 1.2 mg, 9.0 ± 5.4 mg and 6.7 ± 4.4 mg, respectively. For purposes of this comparison, beta-carotene is a suitable model of how lutein +

zeaxanthin intake is calculated. Both methods are generally considered to be accurate and there is no ultimate reference standard for comparison.

4.4 Summary of Estimates of Zeaxanthin Intake

Using 24-hour dietary recall methodology in a nationally-representative sample of the US population, average zeaxanthin intake ranged from 151-213 μg per day depending on age and gender grouping (Mohammedshah et al., 1999).

Using diet history methodology in a sample of subjects from California, Utah and Minnesota (n = 4403), average zeaxanthin intake ranged from 155-169 μg per day depending on gender and case-control grouping (Slattery et al., 2000).

Using FFQ methodology in a nationally-representative sample of the US population (n = 16,502), average lutein + zeaxanthin intake ranged from 1860-2210 μg per day depending on gender and date of the survey. At a lutein : zeaxanthin ratio of 5:1 (see Section 4.2.1), estimated average zeaxanthin intake would range from 310-368 μg per day (Nebeling et al., 1997).

Using FFQ methodology in seven, non-nationally representative samples of subjects (n = 576 – 77466), estimates of median intake of lutein + zeaxanthin ranged from 811-3182 μg per day while mean intake estimates ranged from 962-3087 μg per day depending on the study. At a lutein : zeaxanthin ratio of 5:1 (see Section 4.2.1), estimated median zeaxanthin intake would range from 135-530 μg per day and estimated mean zeaxanthin intake would range from 160-515 μg per day (Table 9).

Table 12: The Range Of Zeaxanthin Intakes Reported in Various Studies Categorized by Dietary Intake Methodology, Use of Nationally-Representative Population Samples, or Whether Zeaxanthin Intake Was Calculated Directly or Estimated From Lutein + Zeaxanthin Intake.

Methodology	National Sample?	Estimated Intake?	Zeaxanthin Intake Range (μg /day)
24-hour Recall	Yes	No	151-213
Diet History	No	No	155-169
FFQ	Yes	Yes	310-368
FFQ	No	Yes	160-515

4.5 Examples of High Zeaxanthin Intakes

A number of studies have reported that some people regularly consume zeaxanthin in amounts much higher than the average intake estimates reviewed in Section 4.1.4.

4.5.1 Studies Reviewed in Previous Sections

Zeaxanthin intake was estimated in data reporting lutein+zeaxantin combined intake by using a ratio of 5:1 lutein to zeaxanthin, as per section 4.2.1.

In Seddon et al. (1994), median lutein + zeaxanthin intake for the highest quintile of intake was 5757 μg per day. At a lutein : zeaxanthin ratio of 5:1, estimated median zeaxanthin intake would be 960 μg per day.

In Tucker et al. (1999), median lutein + zeaxanthin intake for the highest quintile of intake was approximately 6000 μg for women and 4800 μg for men (estimated from graphed data). At a lutein : zeaxanthin ratio of 5:1, estimated median zeaxanthin intake would be 1000 and 800 μg per day, respectively.

In Nebeling et al. (1997), black men in 1987 consumed 3.79 ± 0.25 mg lutein + zeaxanthin and black women in 1987 consumed 3.60 ± 0.14 mg (n = 1190 total blacks). At a lutein : zeaxanthin ratio of 5:1, estimated mean zeaxanthin intake would be 632 and 600 μg per day, respectively.

In Brown et al. (1999), median lutein + zeaxanthin intake for the highest quintile of intake was 6871 μg per day. At a lutein : zeaxanthin ratio of 5:1, estimated median zeaxanthin intake would be 1145 μg per day.

In Chasen-Taber et al. (1999), median lutein + zeaxanthin intake for the highest quintile of intake was 11685 μg per day. At a lutein : zeaxanthin ratio of 5:1, estimated median zeaxanthin intake would be 1948 μg per day.

4.5.2 Studies from Outside the US

Zeaxanthin intake was estimated in data reporting lutein+zeaxantin combined intake by using a ratio of 5:1 lutein to zeaxanthin, as per section 4.2.1.

Francheschi et al. (2000) studied 304 esophageal cancer patients and 743 control subjects in Northern Italy. Dietary intake was measured using FFQ, and lutein + zeaxanthin intake was calculated using a local food composition database (S. Salvini et al., unpublished observations). Mean lutein + zeaxanthin intake among all subjects was 5.2 ± 2.8 mg per day. At a lutein : zeaxanthin ratio of 5:1, estimated median zeaxanthin intake would be 867 μg per day.

Botterweck et al. (2000) studied 282 gastric cancer patients and 3123 healthy controls in the Netherlands Cohort Study. Dietary intake was measured using FFQ, and lutein + zeaxanthin intake was calculated using the Dutch Food Composition Table (unpublished). Median lutein + zeaxanthin intake in the highest quintile of intake was 3.81 mg per day. At a lutein : zeaxanthin ratio of 5:1, estimated median zeaxanthin intake would be 635 μg per day.

Le Marchand et al. (1995) studied 82 Fijians, 88 Fiji Indians, 34 Hawaii Filipinos, 98 Cook Islanders and 61 Hawaii Caucasians (among others) in an ecological study of lung cancer in the South Pacific. Dietary intake was measured by diet history, and lutein + zeaxanthin intake was calculated using data from Mangels et al. (1993). Mean daily intake of lutein + zeaxanthin was 25672 μg among Fijians, 19503 μg among Fiji Indians, 8537 μg among Hawaii Filipinos, 6155 μg among Cook Islanders and 5237 μg among Hawaii Caucasians. At a lutein : zeaxanthin ratio

of 5:1, estimated mean zeaxanthin intake would be 4279 µg, 3251 µg, 1423 µg, 1026 µg and 873 µg, respectively.

The studies are summarized in Table 13.

In summary, several studies demonstrate that non-representative samples of US and non-US populations consume median or mean intakes between 600-4279 µg zeaxanthin per day (estimated) without apparent ill effects.

Table 13: Estimated Mean and Median Intakes of Zeaxanthin in Selected Studies Observing High Lutein + Zeaxanthin Intakes in Some Groups

Study Name	Sample Size	Lutein + Zeaxanthin Intake (µg /day)	Estimated Zeaxanthin Intake (µg /day)	Group	Reference
Studies in US Populations					
Eye Disease Case Control Study	876	5757	960	Highest Quintile Median	Seddon et al., 1994
Framingham Heart Study	576	6000	1000	Estimated Highest Quintile Median (women)	Tucker et al., 1999
Framingham Heart Study	576	4800	800	Estimated Highest Quintile Median (men)	Tucker et al., 1999
National Health Interview Survey 1987	1190 (all blacks)	3790	632	Mean Intake Among Black Men	Nebeling et al, 1997
National Health Interview Survey 1987	1190 (all blacks)	3600	600	Mean Intake Among Black Women	Nebeling, et al., 1997
Health Professionals Follow-up Study	36644	6871	1145	Highest Quintile Median	Brown et al., 1999
Nurse's Health Study	77466	11685	1948	Highest Quintile Median	Chasan-Taber et al., 1999
Studies from Outside the US					
Northern Italy	304 cases 743 controls	5200	867	Mean Intake	Francheschi et al., 2000
Netherlands Cohort Study	282 cases 3123 controls	3810	635	Highest Quintile Median	Botterweck et al., 2000
Fiji	82	25672	4279	Mean Intake	Le Marchand et al., 1995
Fiji Indian	88	19503	3251	Mean Intake	Le Marchand et al., 1995
Hawaii Filipino	34	8537	1423	Mean Intake	Le Marchand et al., 1995
Cook Island	98	6155	1026	Mean Intake	Le Marchand et al., 1995
Hawaii Caucasian	61	5237	873	Mean Intake	Le Marchand et al., 1995

4.6 Discussion

Ten studies have been reviewed that report daily intake of lutein and/or zeaxanthin, two natural carotenoid constituents of human food. In general, the methods used to determine intake (24-hour dietary recall, diet history or FFQ) involved answering questions designed to determine the type and amount of foods consumed in the past. After determining the amount and type of foods consumed, the intake of lutein and/or zeaxanthin was calculated using published food composition tables.

The FFQ method (7 separate studies) resulted in exposure estimates that were somewhat higher than those estimates derived from a 24-hour dietary recall source. Higher carotenoid intake estimates from FFQ versus 24-hour dietary recall have been reported previously (Katsouyanni et al., 1997), and both methods are widely considered to be accurate.

Two studies were conducted in representative samples of the US population (Mohamedshah, et al., 1999; Nebeling et al., 1997) and found average zeaxanthin intake estimates to range from 151-368 μg per day depending on intake estimation methodology. These estimates are supported by a similar range of average zeaxanthin intakes (155-515 μg per day) observed in eight studies in non-representative groups of subjects.

Several studies demonstrate that non-representative samples of US and non-US populations consume median and mean intakes between 151-4279 μg zeaxanthin per day (estimated) without apparent ill effects.

4.7 Conclusion

The US population consumes on average between 0.1 and 0.4 mg of zeaxanthin per day. Intakes between 0.151 and 4.3 mg of zeaxanthin per day have been observed in healthy populations worldwide.

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Chapter 5: Toxicology

Toxicology Summary

Zeaxanthin

Preclinical safety assessment

5.1 Genotoxicity

Five different *in vivo* or *in vitro* assays were performed: Design and results of the studies are summarized below. It can be concluded that zeaxanthin per se and metabolites formed by rat liver enzymes have no genotoxic potential.

5.1.1 Ames tests

Mutagenicity Evaluation of Zeaxanthin, [redacted] in the Salmonella / Microsome Assay (Ames Test). [redacted]

The [redacted] of zeaxanthin was evaluated for mutagenic activity in the Ames assay using the plate incorporation and the preincubation method. Seven Salmonella typhimurium standard tester strains were employed (TA 1535, TA 1537, TA 1538, TA 97, TA 98, TA 100 and TA 102) with and without an exogenous metabolic activating enzyme system (S9-Mix) derived from livers of phenobarbital/benaphtoflavone treated male rats. Due to the strong precipitation of the test compound in the aqueous medium, 1500 µg/plate was chosen as highest dose level. There was no increase of the numbers of mutants in any of the tester strains while the positive controls verified the sensitivity of the strains and the activity of the S9-mix. (1)

In one very early laboratory batch of [redacted] zeaxanthin, a positive result was found in the Ames test. It was shown that zeaxanthin in itself is not mutagenic; rather, degradation products formed during exposure of crystalline zeaxanthin to air and light are responsible for the mutagenic activity (2). In addition, it was considered by the author that formulation materials did scavenge the mutagenic activity of degraded crystalline zeaxanthin, thus protecting further for occurrence of mutagenic activity [redacted] zeaxanthin. [redacted]

The formulation of the marketing product includes the addition of antioxidants, which prevent degradation. Mutagenic activity of the marketing formulated product can be excluded on the basis of the reported investigations.

In GLP-compliant mutagenicity tests, no mutagenic activity was found.

5.1.2 V79 assay

Gene Mutation Assay in Cultured Mammalian Cells with [redacted] Zeaxanthin) (V79/HGPRT Test). [redacted]

In the gene mutation assay in cultured mammalian cells (V79) zeaxanthin [redacted] zeaxanthin; active ingredient) was tested for its ability to induce gene mutations at the HGPRT (Hypoxanthine Guanine Phosphoribosyl Transferase) locus in the established cell line V79, derived form Chinese hamster lung cells. Treatment with [redacted] µg to [redacted] µg/ml [redacted] mmol/L) did not induce mutations to 6-Thioguanine resistance in V79 cells in vitro, neither in the absence nor in the presence of a rat liver activation system. (3)

5.1.3 Unscheduled DNA synthesis assay

Unscheduled DNA Synthesis Assay with the Carotenoid [redacted] Zeaxanthin) Using Primary Cultures of Rat Hepatocytes. [redacted]

The ability of zeaxanthin [redacted]-zeaxanthin; active ingredient) to induce DNA damage was tested by the Unscheduled DNA Synthesis assay (UDS test) as measured by the incorporation of radio-labeled nucleotides into non-replicated DNA of freshly isolated rat hepatocytes. A 20-hour exposure to [redacted] µg to [redacted] µg [redacted] per ml did not induce DNA repair synthesis in primary cultures of rat hepatocytes. (4)

5.1.4 Clastogenesis Assay in Human Peripheral Lymphocytes

Chromosome Analysis of Human Peripheral Blood Lymphocytes Exposed In Vitro to the Carotenoid [redacted] Zeaxanthin) in the Presence and Absence of a Rat Liver Activation System. [redacted]

The potential clastogenic activities of zeaxanthin [redacted] zeaxanthin; active ingredient) *in vitro* was assessed using human peripheral blood lymphocytes as target cells in the presence and absence of rat liver activating enzyme system (S9-mix). Under the experimental conditions described, neither zeaxanthin nor any of its metabolites induced chromosomal aberrations in human peripheral blood lymphocytes. (5)

5.1.5 *In Vivo* Mouse Micronucleus Assay

Mutagenicity Studies with [redacted] in Mammalian Systems. 1. The Micronucleus Test in the Mouse. [redacted]

Zeaxanthin was tested in the in vivo micronucleus assay in mice. Oral doses of [redacted] and [redacted] mg, zeaxanthin [redacted] i.e. [redacted] mg pure substance, per kg bodyweight, were administered 30 and 6 hours prior to sacrifice. There was no increase of micronuclei. It was concluded that under the conditions of the study zeaxanthin did not induce chromosome breaks nor mitotic non-disjunctions in mouse bone marrow cells at doses up to [redacted] mg zeaxanthin /kg body weight. (6)

5.2 Acute toxicity

Acute Toxicity Studies with Zeaxanthin and its Precursors. [redacted]

Acute toxicity studies with zeaxanthin were performed in rats and mice. All mice and rats survived a single oral dose of up to [redacted] mg/kg in rats and [redacted] mg/kg in mice. The LD_{50} values in rats and mice, therefore, were greater than [redacted] and [redacted] mg/kg body weight, respectively (7).

5.3 Subchronic Toxicity Studies

Based on preliminary study data (8, 9, 10, 11), subchronic toxicity studies with zeaxanthin were performed in mice (12), rats (13, 14) and dogs (15).

5.3.1 Tolerance Study with [redacted] (Zeaxanthin as [redacted]) Administered Orally as a Feed Admixture to Mice over 13 Weeks.

[redacted]

A 13-week oral toxicity study was performed in mice with a [redacted] formulation of zeaxanthin ([redacted]), and it was administered as a feed admixture. Groups of 10 male and 10 female mice were dosed at nominal [redacted] mg zeaxanthin/kg body weight/day. By addition of placebo [redacted] all 4 groups received similar amounts of [redacted]. In addition there was a beadlet control group. There were no treatment related findings at the ophthalmoscopic examinations and in the hematological and biochemical investigations in the blood. No discoloration of adipose tissue was reported. No toxic effects attributable to zeaxanthin, or the matrix of [redacted] were observed. Findings at necropsy and histopathological examination of tissues revealed no treatment-related changes. The no observed adverse effect level (NOAEL) of [redacted] was > [redacted] mg/kg body weight/day in mice. (12)

5.3.2 Tolerance Study with [redacted] (Zeaxanthin as [redacted]) Administered Orally as a Feed Admixture to Rats over 13 Weeks

[redacted]

A 13-week oral tolerance study in rats was conducted with a [redacted] formulation of zeaxanthin ([redacted]), administered as a feed admixture. Groups of 16 male and 16 female rats were dosed at nominal [redacted] mg zeaxanthin/kg body weight/day). By addition of placebo [redacted] all 4 groups received the similar amounts of [redacted] and a "[redacted]" control group was included. There was a slight reduction of body weight gain, especially in the high dose rats, due to selective consumption of feed and avoidance of the [redacted], which occurred after several weeks. Therefore, the compound intake was reduced to approximately 40 % (females) and 65 % (males) towards the end of the study in the [redacted] mg/kg group. Ophthalmoscopic examinations, urinalysis, hematological and biochemical investigations in the blood did not show any treatment-related changes. In contrast to a later 13-week toxicity study in rats (14) and dogs (15) no discoloration of adipose tissue was reported in the current study. Findings at necropsy and histopathological examination of organs revealed no treatment-related changes. The no observed adverse effect level (NOAEL) [redacted] was > [redacted] mg/kg in rats. (13)

5.3.3 A 13-Week Toxicity Study with [redacted] in the Rat P.O. (feed admix).

[redacted]

A 13-week oral tolerance study in rats was performed with a [redacted] formulation of zeaxanthin [redacted]. Groups of 16 male and 16 female rats were given the test article as a feed admixture to achieve target doses of [redacted] mg/kg body weight/day of zeaxanthin. All treatment groups received similar amounts of [redacted], by adjusting the diet of the low and mid dose groups with control [redacted]. There was no effect on food intake and body weight. Yellow-orange discoloration of the feces was seen in all rats treated with the test article, especially at the high dose. No treatment-related changes in hematological and clinical chemistry parameters were reported. Urine pH values were slightly decreased in male rats of all dose-groups. At necropsy, a slight orange discoloration of the adipose tissue was reported in all treated animals. There were no treatment related changes in organ weights and microscopical examination of the tissues. Under the conditions of this study, the no observed adverse effect level (NOAEL), therefore, was >[redacted] mg/kg body weight/day, because of the discoloration is not considered to be an adverse effect. (14)

5.3.4 13-Week Tolerance Study of [redacted] Administered Orally in Capsules to Dogs.

[redacted]

A 13-week subchronic toxicity study in dogs was performed with a [redacted] formulation of zeaxanthin. Zeaxanthin [redacted]) were incorporated into feed pellets and fed to groups of 3 male / 3 female beagle dogs to achieve intake of the active ingredient of [redacted] mg/kg (males) and [redacted] mg/kg (females), respectively. (This corresponds to test article concentrations in feed of [redacted], respectively. The amount of beadlets present in the feed cubes was kept similar for all treatment groups.)

No treatment-related toxicity was observed throughout the study. The test article was found to strongly discolorate and to slightly soften the feces, particularly in the high dose group. No treatment related findings were reported in the ophthalmologic examination, urinalysis, hematological and serum clinical chemistry investigations. At necropsy, male dogs from the mid- and high-dose groups showed slight to moderate discoloration (yellow to reddish) in the adipose tissue. There were no treatment-related microscopical findings in the examination of tissues. The no observed adverse effect level (NOAEL) in this study was >[redacted] mg/kg body weight/day because the discoloration of the adipose tissue was considered not to be an adverse effect. (15)

5.4 Reproduction Toxicity

The teratogenicity of zeaxanthin was studied in rats and rabbits.

5.4.1 Embryotoxicity and Teratogenictiy Study in Rats with Oral Administration (Feed Admix) of [redacted] Zeaxanthin. Segment II-Teratological Study with Postnatal Evaluation.

[redacted]

In a segment II teratology study in rats, zeaxanthin was administered at doses of [redacted] and [redacted] mg/kg/day orally as a feed admixture, in a [redacted] formulation, from day 7 through day 16 of gestation (16).

5.4.2 Embryotoxicity and Teratogenicity Study in Rabbits with Oral Administration of [redacted], Zeaxanthin. Segment II-Teratological Study.

[redacted]

In a segment II teratology study in rabbits zeaxanthin was fed at doses of [redacted] mg/kg orally in [redacted] from day 7 through day 19 of gestation (17).

In both species, rats and rabbits, there were no treatment-related maternal death and no signs of maternal toxicity. Under these conditions, zeaxanthin was neither embryotoxic nor teratogenic in rats and rabbits at doses up to [redacted] mg/kg/day and [redacted] mg/kg/day, respectively.

5.5 Chronic Toxicity

5.5.1 [redacted] and [redacted]: Combined 52-Week Oral (Gavage) Pilot Toxicity Study with [redacted] Carotenoids in the Cynomolgus Monkey.

(Testing Facility: [redacted]

The purpose of the study was to assess the chronic toxicity of zeaxanthin and lutein in primates. and to determine the potential for crystal formation in the retina. The cynomolgus monkey has been shown to be an excellent model to investigate the induction and dose-dependency of carotenoid crystal formation in the retina (18, 19, 20)

Twenty four cynomolgus monkeys were distributed into 5 treatment groups and treated daily, by gavage, for one year. The treatments were: [redacted]. There were 2 males and 2 females in each group. The zeaxanthin [redacted] mg/kg/day [redacted] groups had an additional male and female monkey, which were sacrificed after 6 months of treatment. Both, zeaxanthin a[redacted] were each in a [redacted] formulation. The complete final report for this study is presented in Volumes V, VI and VII (21). In addition, a comprehensive overview on eye examinations from this study, is presented on Volume V (22).

All animals survived the treatment period. All animals in the [redacted] mg/kg/day group of zeaxanthin showed orange/yellow discolored feces from day 2 of the study onwards. There was no effect on overall mean body weight gain and on overall group mean food intake in animals of all treated groups.

There were no changes in ECG and blood pressure data, which could be regarded as being related to administration of zeaxanthin throughout the treatment period. There were no treatment related changes in hematological and serum clinical chemistry parameters, nor changes in urine parameters. There were no organ weight changes, which could be associated with the test article

treatment. Most of the animals showed dark-yellow colored mesenteric fat at interim sacrifice and gold-yellow mesenteric fat at the terminal sacrifice. This finding is considered to be related to the color of the compound. At histopathological examinations, no treatment-related findings were reported (21,22).

Ophthalmic Exams - Ophthalmic examinations were performed on the monkeys by two independent examiners. Both eyes of all animals were clinically examined by [redacted] Indirect ophthalmoscopic examinations were performed using the Bonnoskop and direct ophthalmoscope and a contact lens biomicroscope. Overall, based on [redacted] ophthalmic examination findings, it was concluded that there were no adverse findings that were considered to be related to treatment and there was no evidence for crystalline deposits in the retina of treated monkeys (21,22).

Additional evaluations by [redacted], Director of Research, [redacted] were performed using the ophthalmic slit lamp biomicroscope in combination with wide-field corneal contact fundus lenses. The results of [redacted] examinations showed clearly that there were no crystalline deposits of any sort, nor were there any such inclusions that were in any way similar to those that have been seen in humans or in Cynomolgus monkeys ingesting high dosages of canthaxanthin. There were some retinal findings often seen in the human and primate retina, however, none of these were considered to be related to treatment (21,22).

Electroretinography (ERG) - Electroretinography (ERG) was performed in all animals once pre-dose and during Weeks 25 to 26, Weeks 38 to 39, and 51 to 52 of treatment. There were no treatment-related effects in electroretinograms, which is considered a very sensitive procedure to detect early signs of generalized retinal degeneration (23).

Eye Pathology. Whole-mounts of retinas from the right eyes were used for microscopic investigations of with light or confocal microscopy. Maculas were punched out with a 7 mm trephine before mounting them on slides and the peripheral remaining parts of the retinas were flat-mounted and investigated under the polarization microscope separately. Semiquantitative analysis of inclusions was performed by screening the flat-mounted retinas of the right eyes under polarized light with using a Zeiss Axioplan. In addition, all maculas were investigated using a confocal microscopic system. Routine histopathology of paraffin sections from retinal periphery was also performed (22,24).

The routine histopathological investigation of paraffin sections from retinal periphery did not show any differences between treated or control animals.

It was concluded that there were no treatment-related toxicological changes in the eyes noted under the conditions of this study. Polarizing inclusions were observed in the macula of monkeys, which were not related to zeaxanthin [redacted] treatment. The incidence and grade of the inclusions in the maculas of the monkeys was not treatment or dose-related. The inclusions differed clearly from crystals observed after long-term treatment at high doses of canthaxanthin. The canthaxanthin crystals were strongly dose-dependent, occurred predominantly in the peripheral retina and exhibited crystalloid morphology and larger size. In contrast, inclusions in the current study were restricted to the fovea, very small and showed no typical crystalline morphology. The nature of the observed polarizing structures remains unknown. Since they were also observed in control animals with naturally yellow macula, a physiological function may be hypothesized.

[redacted] **Zeaxanthin Determinations in Retina & Lens by HPLC** - Determination of [redacted] zeaxanthin in retina and lens was made by HPLC. Dose-related increases in [redacted] content was observed after treatment with [redacted] in central retina (macula/parafovea) and peripheral retina, and lens. Animals treated with zeaxanthin [redacted] showed a dose-related increase in zeaxanthin content in the peripheral retina. In central retina and lens, zeaxanthin content was markedly increased in animals of the high dose group. Levels in the low dose group were comparable to those determined in the placebo group. Elevated zeaxanthin concentrations were observed in central retinas of animals treated with [redacted] (25).

Overall, there was no clinical and no morphological evidence for treatment-related adverse changes in the eyes of cynomolgus monkeys during or after 52 weeks treatment with [redacted] (zeaxanthin) or [redacted] both as a [redacted] formulation. Specifically, there was no evidence for crystal formation in the eyes of treated monkeys.

5.6 Skin Sensitization

Determination of Allergenicity of Colorants Used in Products of the Pharmaceutical, Cosmetic and Nutrition Industries in Guinea Pigs. [redacted]

An Optimization Test (according to Maurer) was performed with zeaxanthin in albino guinea pigs of both sexes. No signs of irritation were observed. (26)

5.7 Absorption, Distribution, Metabolism and Excretion (ADME)

5.7.1 Zeaxanthin Balance Studies.

[redacted]

A distribution study with [redacted]-zeaxanthin [redacted]-zeaxanthin) was done in male rats, after a pretreatment (feeding) with zeaxanthin-poor* or Zeaxanthin-enriched diet [redacted] and subsequent single administration of [redacted]-zeaxanthin in a liposomal preparation. One day after dosing, the amount of radioactivity excreted in the urine was almost twice as high as the amount remaining in the carcass (excluding contents in the stomach, small intestine and large intestine). Concomitant with this excretion in the urine, a decrease of radioactivity was found in the tissues of the rats. The pattern of distribution in the tissues and of excretion was similar for rats pre-fed with zeaxanthin-poor and those with zeaxanthin-enriched diet. After one day, about 1/3 of the administered radioactivity was still present in the body and

* In the basic rat diet, [redacted] of Zeaxanthin was detected

GI-tract. After 1 week, less than [redacted] was still present in the body and less than [redacted] in the body and the digestive tract. The urine contents and the amount absorbed tended to be lower for animals in the zeaxanthin-poor diet. It was concluded that the radioactivity from [redacted] zeaxanthin is rapidly depleted from the body and the GI-tract of rats. (27)

5.7.2 Zeaxanthin Distribution Study in Rats.

[redacted]

A study was performed to investigate zeaxanthin distribution in rats fed a zeaxanthin-enriched diet. Male rats received a diet containing [redacted] mg or [redacted] mg zeaxanthin/kg feed (approximately [redacted] mg or [redacted] mg/kg/day body weight), for five weeks, which was prepared by mixing a zeaxanthin [redacted] formulation in the ground feed. A dose-dependent accumulation of zeaxanthin was found, with the exception of the thyroid gland and the eye, where all levels stayed below detection limit. Highest concentrations were found in the small intestine and spleen, followed by liver, fat and adrenal glands. There was a pronounced decrease of zeaxanthin concentration during a subsequent 5-week period with zeaxanthin-free diet. (28)

5.7.3 Radioactivity in Expired Air During Zeaxanthin Balance Studies Compared to Previous Findings for Canthaxanthin and Astaxanthin with Rats.

[redacted]

In balance studies with a liposomal preparation of [redacted]-zeaxanthin in male rats, about [redacted] % of the applied dose, i.e. about [redacted]% to [redacted] % of the absorbed dose was measured in the expired air during the first 24 hours after administration (23). Contribution of respiration in the excretion of radioactivity was considerably higher in the case of zeaxanthin when compared to previous studies with astaxanthin and canthaxanthin. Absorption (biliary excretion not considered) varied from around [redacted] % to around [redacted] % (27, 29).

5.8 Conclusions

The preclinical safety of zeaxanthin has been investigated in a series of toxicity studies including a battery of *in vivo* and *in vitro* mutagenicity studies, acute toxicity, subchronic toxicity in rats, mice and dogs, teratogenicity in rats and rabbits and a chronic toxicity study in primates. No treatment-related toxicity findings were found. The only treatment-related changes identified were discolorations of fatty tissues and feces, which are not considered to be adverse effects.

The toxicity testing of zeaxanthin was performed with test materials from different production processes and various product formulations were used (i.e. [redacted]). Due to the fact that the different toxicity studies did not exhibit different toxicities, it is considered that the different batches did not differ from each other.

The original acute toxicity studies were performed prior to the implementation of GLP. A repetition of acute toxicity in rats just for compliance with current guidelines (doses up to [redacted] mg/kg) would not reveal new insights since the reported LD_{50} values were greater than [redacted] and [redacted] mg/kg in mice and rats, respectively.

Subchronic (13-week) toxicity studies with zeaxanthin were performed in mice, rats and dogs. The no observed adverse effect level (NOAEL) of zeaxanthin in mice was > [redacted] mg/kg body weight/day. The no observed adverse effect level (NOAEL) of zeaxanthin in both subchronic studies in rats was > [redacted] mg/kg. Finally, the no observed adverse effect level (NOAEL) from a subchronic study in dogs study was > [redacted] mg/kg body weight/day (the discoloration of the adipose tissue was considered not to be an adverse effect).

The teratogenicity of zeaxanthin was studied in rats and rabbits. In both species, rats and rabbits, there were no treatment-related maternal death and no signs of maternal toxicity. Under these conditions, zeaxanthin was neither embryotoxic nor teratogenic in rats and rabbits at doses up to [redacted] mg/kg/day and [redacted] mg/kg/day, respectively.

A chronic toxicity study was carried out with monkeys, which were fed with doses of zeaxanthin up to [redacted] mg/kg bw/day. No treatment-related effects were observed. This study provides the NOAEL for assessment of risk from anticipated exposure.

ADME studies revealed that zeaxanthin was rapidly leaving the body and gastrointestinal tract of male rats after single dose treatment. Repeated dose feeding of zeaxanthin resulted in a dose-dependent accumulation of zeaxanthin in some tissues of male rats, which is clearly decreased during a reversibility period.

The safety package provides an adequate database for which an assessment of safety to zeaxanthin can be estimated.

The lowest no adverse effect level (NOAEL) was from a chronic toxicity study carried out with monkeys. At doses of zeaxanthin up to [redacted] mg/kg bw/day (in diet) for one year, no treatment-related effects were observed. Therefore, the NOAEL for zeaxanthin based on this database is [redacted] mg/kg/day.

5.9 Citations from Chapter 5

(These citations will be made available upon request)

1 [redacted] Mutagenicity Evaluation of Zeaxanthin, [redacted] in the Salmonella / Microsome Assay (Ames Test). [redacted]

2 [redacted]. Internal Communication: [redacted]

3 [redacted] Gene Mutation Assay in Cultured Mammalian Cells with [redacted] Zeaxanthin) (V79/HGPRT Test). [redacted]

4 [redacted]: Unscheduled DNA Synthesis Assay with the Carotenoid [redacted] Zeaxanthin) Using Primary Cultures of Rat Hepatocytes [redacted]

5 [redacted] Chromosome Analysis of Human Peripheral Blood Lymphocytes Exposed In Vitro to the Carotenoid [redacted] Zeaxanthin) in the Presence and Absence of a Rat Liver Activation System. [redacted]

6 [redacted] Mutagenicity Studies with [redacted] in Mammalian Systems. 1. The Micronucleus Test in the Mouse. [redacted]

7 [redacted]. Akute Toxizitätsversuche mit zeaxanthin und dessen Vorstufen (Acute Toxicity Studies with Zeaxanthin and its Precursors). [redacted]

8 [redacted] Orale und I.P. 10-Tage-Toxizitaetsversuche an Maeusen und Ratten (Oral and Intraperitoneal 10-Day Toxicity Studies in Mice and Rats). [redacted]

9 [redacted] Vergleichende Pruefung im 5-tage-Toxizitaetsversuch an maennlichen und weiblichen Ratten (Comparative Testing in a 5-Day Toxicity Study in Male and Female rats). [redacted]

10 [redacted]: Vergleichende Pruefung im 5-tage-Toxizitaetsversuch an maennlichen und weiblichen Ratten (Comparative Testing in a 5-Day Toxicity Study in Male and Female Rats). [redacted]

11 [redacted]: Orale 10-Tage-Toxizitaetsversuche an Maeusen und Ratten mit 006 (Oral 10-Day Toxicity Studies in Mice and Rats with 006). [redacted]

12 [redacted] Tolerance Study with [redacted] (Zeaxanthin as [redacted]) Administered Orally as a Feed Admixture to Mice over 13 Weeks [redacted]

13 [redacted] Tolerance Study with [redacted] (Zeaxanthin as [redacted] Administered Orally as a Feed Admixture to Rats Over 13 Weeks. [redacted]

14 [redacted] A 13-Week Toxicity Study with [redacted] in the Rat P.O. (Feed Admix). [redacted]

15 [redacted] 13 Week Tolerance Study of [redacted] Administered Orally in Capsules to Dogs. [redacted]

16 [redacted] Embryotoxicity and Teratogenictiy Study in Rats with Oral Administration (Feed Admix) of [redacted] Zeaxanthin. Segment II-Teratological Study with Postnatal Evaluation. [redacted]

17 [redacted] Embryotoxicity and Teratogenicity Tudy in Rabbits with Oral Administration of [redacted] Zeaxanthin. Segment II-Teratological Study. [redacted]

18 Arden G B, Barker F M. Canthaxanthin and the Eye: A Critical Ocular Toxicological Assessment. J Toxicol-Cut and Ocular Toxicol, 10: 115-155, 1991 (in Volume III, Appendix 4).

19 Goralczyk R, Buser S, Bausch J, Bee W, Zuehlke U, Barker F M. Occurrence of Birefringent Retinal Inclusions in Cynomolgus Monkeys After High Doses of Canthaxanthin. *Invest Ophthalmol Vis Sci* 38: 741-52, 1997 (in Volume III, Appendix 4).

20 Goralczyk R, Barker F M, Buser S, Liechti H, Bausch J. Dose Dependency of Canthaxanthin Crystals in Monkey Retina and Spatial Distribution of its Metabolites. *Invest Ophthalmol Vis Sci* 41: 1513-22, 2000 (in Volume III, Appendix 4).

21 [redacted]: Combined 52-Week Oral (Gavage) Pilot Toxicity Study with [redacted] Carotenoids in the Cynomolgus Monkey. (Testing Facility: [redacted]

22 [redacted]. Comprehensive Overview on Eye Examinations. [redacted]

23 [redacted]. Additional Evaluation of Electroretinography (ERG) and Expert Commentary. [redacted] In *Amendment to Final Report No. 1.* [redacted] (in Volume VI)

24 [redacted] Pathology Report on Eyes. [redacted] In *Amendment to Final Report No. 1.* [redacted] (in Volume VI).

25 [redacted] Determination of [redacted] Zeaxanthin in Retina and Lens by [redacted] In *Amendment to Final Report No. 1.* [redacted] (in Volume VI).

25 [redacted] Die Bestimmung der Allergenität am Meerschweinchen von Farbstoffen, die in Produkten der pharmazeutischen, kosmetischen und Lebensmittel-Industrie verwendet werden (Determination of allergenicity of colorants used in products of the pharmaceutical, cosmetic and nutrition industries in Guinea pigs). [redacted]

26 [redacted] Zeaxanthin Balance Studies. [redacted]

27 [redacted] Zeaxanthin Distribution Study in Rats. [redacted]

28 [redacted]. Radioactivity in Expired Air During Zeaxanthin Balance Studies Compared to Previous Findings for Canthaxanthin and Astaxanthin with Rats. [redacted]

Attachment 1: Compilation of Data from Preclinical Safety Studies

Mutagenicity

Study	Test System(s)	Strain(s) / Target cells	Formulation	Concentration / Dose	GLP audited	Study period	Ref.
In vitro							
Ames	Salmonella typhimurium	TA 1535, 1537, 1538, 97, 98, 100, 102	[redacted]	[redacted] µg/plate*	Yes	[redacted]	1
HGPRT	Chinese Hamster	V 79 cells	[redacted]	[redacted] µg/ml*	Yes	[redacted]	3
UDS	Rat	Hepatocytes	[redacted]	[redacted] µg/ml*	Yes	[redacted]	4
HLA	Human blood	Peripheral lymphocytes	[redacted]	[redacted] µg/ml*	Yes	[redacted]	5
In vivo							
Micronucleus	Mouse	Bone marrow cells	[redacted]	[redacted] mg/kg p.o.	Yes	[redacted]	6

* = With and/or without metabolic activation by fraction of rat liver microsomes (S-9 mix)

Single dose toxicity

Species	Route		Formulation	LD50 (mg/kg bw)	GLP audited	Study period	Ref.
Mouse	Oral		[redacted]	[redacted]	No	[redacted]	7
Rat	Oral		[redacted]	[redacted]	No	[redacted]	7

Repeated dose toxicity

Species	Route	Duration of treatment	Formulation	Animal Nos. and sex Doses (mg/kg bw/day)	GLP audited	Study period	Ref.
Mouse	Oral (Dietary)	3-Month	[redacted]	10 m / 10 f [redacted]	No	[redacted]	12
Rat	Oral (Dietary)	3-Month	[redacted]	16 m / 16 f [redacted]	Yes	[redacted]	13
Rat	Oral (Dietary)	3-Month	[redacted]	16 m / 16 f [redacted]	Yes	[redacted]	14
Dog	Oral (Dietary)	3-Month	[redacted]	3 m: [redacted] 3 f: [redacted]	Yes	[redacted]	15
Monkey	Oral (Gavage)	12-Month	[redacted]	2 m / 2 f [redacted] 3 m / 3 f [redacted] Interim sacrifice 1 m / 1 f at [redacted] mg/kg/d	Yes	[redacted]	18

Reproduction toxicity

Study	Species	Route	Formulation	Doses (mg/kg bw/day)	GLP audited	Study period	Ref.
Segment II	Rat	Oral	[redacted]	[redacted]	Yes	[redacted]	16
Segment II	Rabbit	Gavage	[redacted]	[redacted]	Yes	[redacted]	17

Skin Sensitization

Study	Species		Formulation		GLP audited	Study period	Ref.
Optimization (Maurer)	Guinea pig		Not given		No	[redacted]	20

Attachment 2: Ingredients in the zeaxanthin formulations

Formulation Compound	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]	[redacted]
[redacted]	X	X	X	X	X ^{14}C	X
[redacted]	X	X	X	X	-	X
[redacted]	X	X	X	X	-	X
[redacted]	X	-	-	X	-	-
[redacted]	X	X	X	X	-	X
[redacted]	-	X	-	-	X	X
[redacted]	X	X	-	X	-	X
[redacted]	X	-	X	X	-	-
[redacted]	X	-	-	-	-	-
[redacted]	-	X	-	-	-	X
[redacted]	-	-	-	-	X	-
[redacted]	-	-	X	-	-	-

*In some mutagenicity studies (references # 1, 3, 4, 5) the material [redacted] denotes the crystalline form.

Chapter 6: Proposed Use and Estimate of Safety

6.1 Restatement of Proposed Use and Labeling

Zeaxanthin is recommended to be consumed as a dietary supplement at a dose of 1 mg per day. This exposure would translate to a dose of 0.017 mg/kg in a 60 kg human or 0.025 mg/kg in a 40 kg human.

Zeaxanthin is a dietary supplement that helps maintain healthy eyesight. This statement has not been evaluated by the Food and Drug Administration. This product is not intended to diagnose, treat, cure, mitigate or prevent any disease.

6.2 Restatement of Existing Exposure

Ten studies have been reviewed that report daily intake of lutein and/or zeaxanthin, two natural carotenoid constituents of human food. In general, the methods used to determine intake (24-hour dietary recall, diet history or FFQ) involved answering questions designed to determine the type and amount of foods consumed in the past. After determining the amount and type of foods consumed, the intake of lutein and/or zeaxanthin was calculated using published food composition tables.

Several studies demonstrate that non-representative samples of US and non-US populations reported median and mean intakes between 0.15 – 4.3 mg (150 – 4300 μg) zeaxanthin per day (estimated) without apparent ill effects. The data showed that the US population consumes on average between 0.1 and 0.4 mg (100 – 400 μg) of zeaxanthin per day. Intakes between 0.15 and 4.3 mg of zeaxanthin per day have been observed in healthy populations worldwide.

Therefore, the highest existing exposure worldwide, or 4.3 mg (4300 μg), is equivalent to about 0.072 mg/kg in a 60 kg human or 0.11 mg/kg in a 40 kg human. This existing exposure level is conservative and is approximately 10 times higher than that estimated for the U.S. population. In addition, the conservative nature of the estimate is inflated further by the use of a basis of 40 kg as the average human bodyweight.

6.3 Summary of Exposure (Anticipated Exposure)

The anticipated exposure would be equal to the Existing Exposure plus the proposed use of the product.

$$\text{Anticipated Exposure} = \text{Proposed Use} + \text{Existing Exposure}$$

$$\text{Anticipated Exposure} = 0.025\,mg/kg + 0.11\,mg/kg$$

$$\text{Anticipated Exposure} = 0.135\,mg/kg$$

This anticipated exposure represents a conservative approximation of the exposure and is approximately 4 times higher than that estimated for U.S. populations. This approximation is further inflated by the use of a basis of 40 kg as the average human bodyweight.

6.4 Restatement of Safety Principles and No Effect Level

The lowest no effect level is 20 mg/kg based on a chronic (52 week oral gavage) study in primates.

6.5 Margin of Safety

6.5.1 Safety Factor Selection

A safety factor is defined as the ratio of the lowest no effect level derived from the safety database to the human daily exposure (both values in mg/kg bodyweight). Traditionally, the safety factor is comprised of: (1) an uncertainty factor that accounts for variation in human sensitivity among populations (intra-human variation), (2) an uncertainty factor that accounts for uncertainties assumed when extrapolating from animal data to humans (interspecies factor) (Lehman and Fitzhugh, 1954), (3) an uncertainty factor that accounts for uncertainties assumed when extrapolating from a less than chronic study (Kokoski *et al.*, 1990), and (4) an uncertainty factor that accounts for uncertainties assumed when extrapolating from a LOAEL to a NOAEL (Dourson *et al.*, 1996). This value is considered a conservative estimate of the magnitude of the uncertainty; lower values derived on a case-by-case basis using a weight-of-evidence approach are likely to provide a more realistic estimate of uncertainty. In addition to the above factors, an additional modifying factor may be utilized to account for additional uncertainty not explicitly included in the traditional factors (such as database quality and completeness).

Of these possible factors, only the first two factors, intra-human variation factor and inter-species factor, are deemed relevant for the zeaxanthin analysis. The critical study selected is a chronic study and the dosage selected a NOAEL; hence, factors for study duration and effect dosage are not needed. A modifying factor was also not utilized in this safety factor determination, as the existing database is reasonably robust in species and toxicity endpoints tested. The database, however, has acknowledged limits in pertinence of exposure route and study quality. For the two selected factors, values of 10 were adopted for each to assure conservatism. Because the lowest no effect level for Zeaxanthin is based on a chronic repeated dose study in monkeys (Pfannkuch F et al. Research Report B-0171423. 11-May-2000), application of these two factors results in a total safety factor of 100.

6.5.2 Calculation of Margin of Safety for the Proposed Use of Zeaxanthin

The Safety factors are calculated as the ratio between the NOEL derived from the safety studies and the anticipated exposure of the material. Therefore:

$$\text{Margin of Safety} = \frac{\text{NOEL from Safety}}{\text{Anticipated Exposure}}$$

$$\text{Margin of Safety} = \frac{20\,mg/kg}{0.135\,mg/kg}$$

$$\text{Margin of Safety} = 148$$

Because the Margin of Safety was calculated to be greater than 100, acceptable regulatory policy would consider the proposed use of zeaxanthin as having an adequate safety margin. In addition, since the approach used to select the largest existing exposure worldwide and the use of 40 kg as the average human body weight, the calculated margin of safety is a particularly conservative estimate.

Therefore, the proposed use of zeaxanthin is considered safe within the confines of regulatory approaches currently acceptable to the scientific community.

6.6 Summary

The proposed use for zeaxanthin in this submission is 1 mg/day.

The greatest existing exposure (from the diet, worldwide) of zeaxanthin found from sources in the scientific literature was 4.3 mg (4300 µg) per day. Therefore, the anticipated exposure (defined as the sum of the use proposed in this submission and the greatest existing exposure) is 0.135 mg/kg per day (in a 40 kg human).

The lowest no effect level determined from a chronic (52 week gavage) study in primates is 20 mg/kg.

The ratio of the no effect level to the anticipated exposure is 148. This margin of safety, greater than 100, is acceptable because of the nature of the study from which the no effect level was derived and the robust nature of the existing safety database on zeaxanthin. In addition, the estimated margin of safety is conservative because of the selected human exposure is the largest found worldwide and the use of a 40 kg average human bodyweight.

Therefore, the proposed use of zeaxanthin is considered safe within the confines of regulatory approaches currently acceptable to the scientific community.

6.7 References from Chapter 6

Dourson M.L., Felter S.P. and Robinson D. Evolution of Science-Based Uncertainty Factors in Noncancer Risk Assessment. *Regulatory Toxicology and Pharmacology.* 24:108-120, 1996.

Kokoski, C.J., Henry, S.H., Lin, C.S., and Ekelman, K. B. Chapter 15 Methods Used in Safety Evaluation in Food Additives (Branen, A.L., Davidson, P. M., and Salminen, S. Editors) pp 570-616, Marcel Dekker, Inc, NY, 1989.

Lehman, A.J. and Fitzhugh, O.G. (1954). 100-Fold Margin of Safety. *Quart. Bull. Assoc. Food Drug Officials, U.S.* 18: 33 - 35.

Appendix 1 References for Chapter 3 (Alpers- Ham)