DEPARTMENT OF HEALTH & HUMAN SERVICES

Public Health Service
Food and Drug Administration

Memorandum

Date: OCT 19 2000

0793 '00 OCT 30 P1:48

From: (Acting) 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:	August 8, 2000
90-Day Date:	November 6, 2000

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 November 6, 2000.

Felicia B. Satchell

Felicia B. Satchell

95S-0316

RPT81

ZEAXANTHIN 5%

Volume 1 of 3

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Chapter 1

The new dietary ingredient

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 New Dietary Ingredient

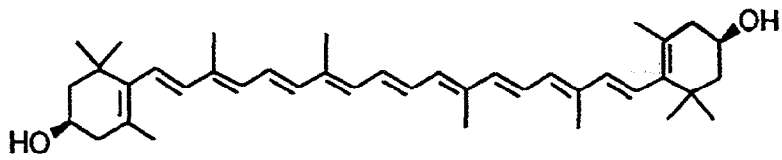
The new dietary ingredient is zeaxanthin and it will be available in a beadlet formulation containing 5% zeaxanthin (Zeaxanthin 5%TG), for the manufacture of dietary supplements. Zeaxanthin is an antioxidant that helps maintain healthy eyesight.

1.3 Description of the New Dietary Ingredient

1.3.1 Chemistry

The new dietary ingredient is zeaxanthin, which belongs to the class of carotenoids called xanthophylls. The chemical structure of zeaxanthin makes it highly soluble in lipids and 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).

1.3.2 Assay and specifications

1.3.3 Manufacturing principles

Scheme

DEPARTMENT OF HEALTH AND HUMAN SERVICES

Public Health Service

Food and Drug Administration
Washington, DC

OCT 19 2000

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 in response to your letter to the Food and Drug Administration (FDA) dated August 3, 2000, making a submission for a new dietary ingredient pursuant to 21 U.S.C. 350b(a)(2) of the Federal Food, Drug, and Cosmetic Act (the Act). Your letter notified FDA of your intent to market a product containing a new dietary ingredient named zeaxanthin. FDA received your submission on August 8, 2000.

21 U.S.C. 350b(a)(2) requires that a manufacturer or distributor of a dietary supplement that contains a new dietary ingredient submit to FDA, at least 75 days before the dietary ingredient is introduced or delivered for introduction into interstate commerce, information that is the basis on which the manufacturer or distributor has concluded that a dietary supplement containing such new dietary ingredient will reasonably be expected to be safe. FDA reviews this information to determine whether it provides an adequate basis for such a conclusion. Under 21 U.S.C 350b(a)(2), there must be a history of use or other evidence of safety establishing that the dietary ingredient, when used under the conditions recommended or suggested in the labeling of the dietary supplement, will reasonably be expected to be safe. If this requirement is not met, the dietary supplement is deemed to be adulterated under 21 U.S.C. 342(f)(1)(B) because there is inadequate information to provide reasonable assurance that the new dietary ingredient does not present a significant or unreasonable risk of illness or injury.

FDA has carefully considered the information in your submission, and the agency has significant concerns about the evidence on which you rely to support your conclusion that the new dietary ingredient zeaxanthin will reasonably be expected to be safe. The information in your submission does not meet the requirements of 21 CFR §190.6(b)(3) because it does not contain a description of the dietary supplement that contains the new dietary ingredient including the level of the new dietary ingredient in the dietary supplement (see 21 CFR §190.6(b)(3)(i)), nor does it describe, in a quantitative manner, the amount to be consumed daily. The submission contains evidence of history of use and other information that you assert is an adequate basis to conclude that the dietary supplement containing the new dietary ingredients will reasonably be expected to be safe. However, the information in the submission is inadequate to make such a determination (see 21 CFR §190.6(b)(4)). The

submission provides insufficient information to enable a determination to be made that the levels and strength of zeaxanthin tested in the studies are relevant to determining whether your product, as formulated and at the expected exposure when used as suggested in labeling, would reasonably be expected to be safe. Furthermore, the submission fails to include the complete results of the unpublished toxicology studies that are needed to fully evaluate the safety of zeaxanthin. The submission fails to elucidate whether the recommended dietary supplement intakes are comparable to the amount of zeaxanthin consumed in a typical diet. This information is necessary to evaluate whether it is appropriate to extrapolate a safe level of supplementation from dietary exposure and whether additive exposure to zeaxanthin would be safe for adults or for children. In addition, the significance of some effects associated with zeaxanthin consumption whose adverse nature is unclear is not addressed.

For the reasons discussed above, the information in your submission does not provide an adequate basis to conclude that zeaxanthin, when used under the conditions recommended or suggested in the labeling of your product, will reasonably be expected to be safe. Therefore, your product may be adulterated under 21 U.S.C. 342(f)(1)(B) as a dietary supplement that contains a new dietary ingredient for which there is inadequate information to provide reasonable assurance that such ingredients do not present a significant or unreasonable risk of illness or injury. Introduction of such products into interstate commerce is prohibited under 21 U.S.C. 331(a) and (v).

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

Sincerely yours,

Felicia B. Satchell

Felicia B. Satchell
(Acting) Director
Division of Standards
and Labeling Regulations
Office of Nutritional Products, Labeling
and Dietary Supplements

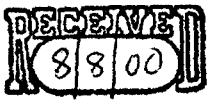
Roche

Vitamins

Tel: 973-257-8325
Fax: 973 257-8414

VIA COURIER

August 3, 2000



Dr. Robert J. Moore
Office of Special Nutritionals (HFS-450)
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 Dr. Moore,

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 1.3.3 " Manufacturing Principles" and Chapter 4 "Toxicology Summary".

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.

The submission is divided in three volumes. Volume one contains the description, chemistry, nutritive effect and safety evaluation. We have included all the references cited; they are organized in alphabetical order and divided into two volumes: Volume two contains the references from A to K and volume three contains the references from L to Z.

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

Sincerely,

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

Enclosure

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

Chapter 2

Zeaxanthin

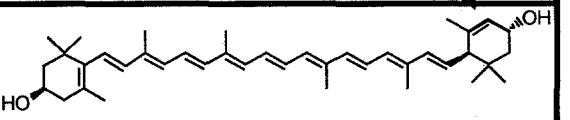
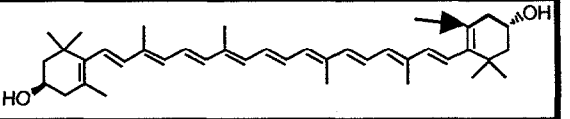
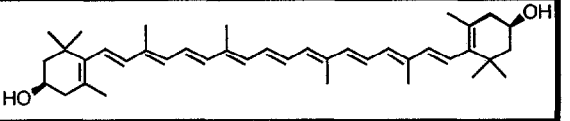
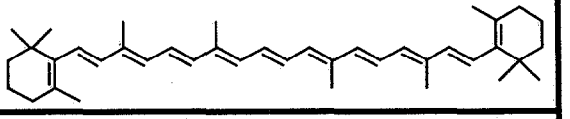
Occurrence in the diet and functionality in the human eye

2.1 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., 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 article will focus on the unique properties of zeaxanthin as well as the properties that it shares with lutein.

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/mm^2)	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.

2.2 Nutritional aspects - zeaxanthin is a constituent of the normal 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

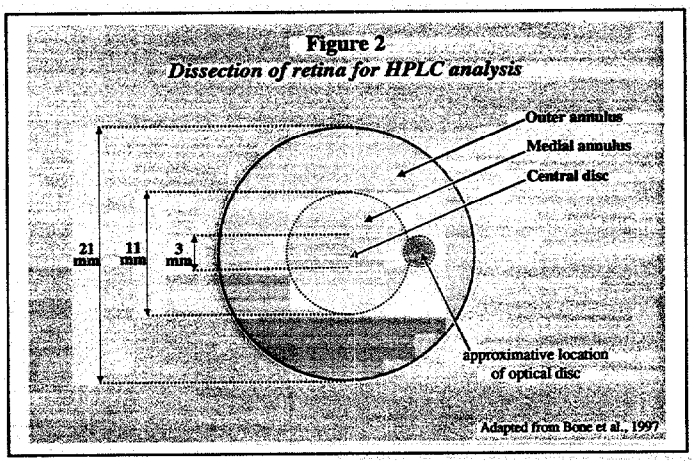
called the macular carotenoids. Given their presence in the retina, a valid question is whether they are involved in the visual cycle as a result of pro-vitamin A activity. No substantial vitamin A activity of macular carotenoids has been detected (Weiser et al., 1993), indicating that they can not provide retinol to the retina and must act in a different mechanistic way.

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 2, many vegetables and fruits contain markedly more lutein than 3R,3'R-zeaxanthin (e.g. kale, spinach, broccoli)

Table 2: 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, 1996a)

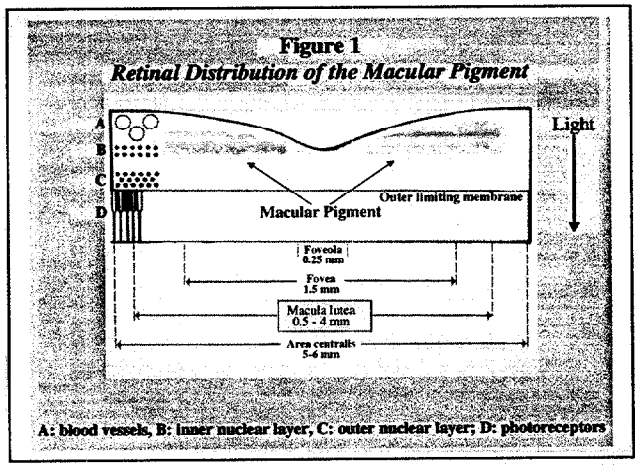
Food	Form	Lutein (µg/100g)	3R, 3'R-Zeaxanthin (µg/100g)	Lutein: Zeaxanthin Ratio
Beans	snap, green, canned, regular pack	616	44	14 : 1
Broccoli	cooked	2203	23	96 : 1
Carrots	baby, raw	335	23	15 : 1
Celery	cooked	242	8	31 : 1
Celery	raw	229	3	77 : 1
Collards	cooked	7825	266	30 : 1
Corn	sweet, yellow, 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	-	5.00	-
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

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 a few plants: corn, oranges, peaches, persimmons, red or orange bell peppers and the small red berry *Lycium barbarum*, "Gou Qi Zi" (Table 1). 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.

2.3 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).

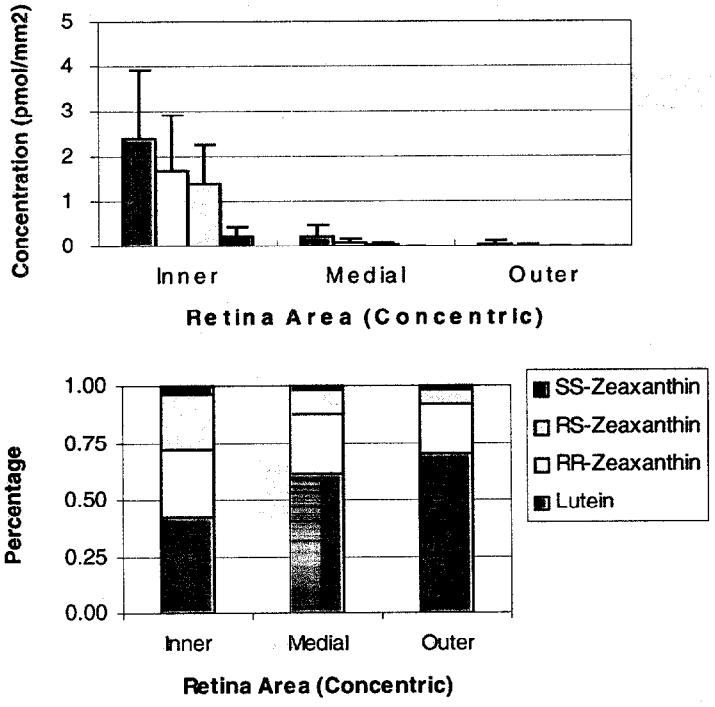


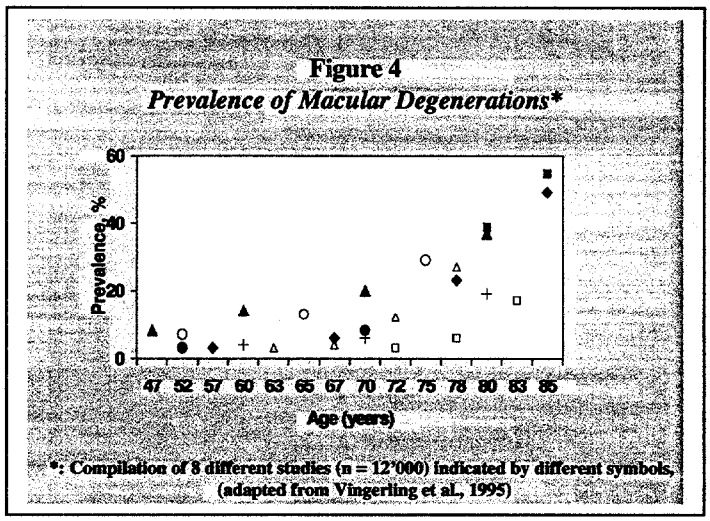
Figure 3: Absolute and relative concentrations of lutein, RR-zeaxanthin, RS-meso-zeaxanthin and SS-zeaxanthin in 16 human retinas (Landrum, et al, 1999)

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 & Balashov (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).

2.4 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.

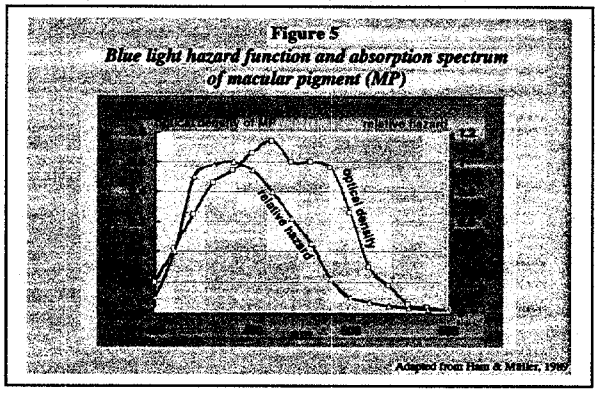
The etiology of AMD is only poorly understood, and both genetic and environmental factors have been hypothesized as playing a role. One of the environmental factors seems to be ocular exposure to sunlight (McCarty & Taylor, 1999) in particular a history of exposure to blue light in the preceding 20 years (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.

2.5 Zeaxanthin and lutein in risk reduction of AMD

2.5.1 The mechanistic basis



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).

2.5.2 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 3 (Schalch et al. 1999).

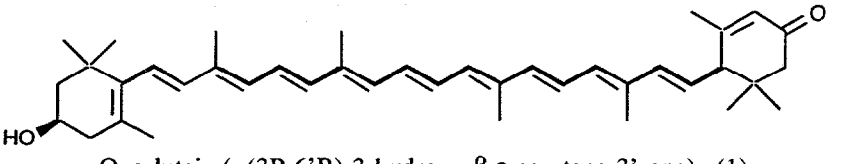
Additional *in vitro* experiments (Mortensen et al, 1997) indicate that zeaxanthin is also more efficient than lutein in neutralizing some (e.g. the nitrogen dioxide radical (NO_2•) or the 2-mercaptoethanol thiyl radical ($HO(CH_2)_2S$•) but not all radical species (e.g. the glutathione thiyl radical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

Table 3

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.

2.6 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.

2.6.1 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 of Gou Qi Zi berries, equivalent to 50 mg of 3R,3'R-zeaxanthin per day (Table 2), 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.

2.6.2 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.

2.6.3 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 [manuscript submitted for publication]), indicating that the metabolism of macular carotenoids in this aquatic animal may be similar to that in humans.

2.6.4 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.

2.7 Investigations in humans

2.7.1 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).

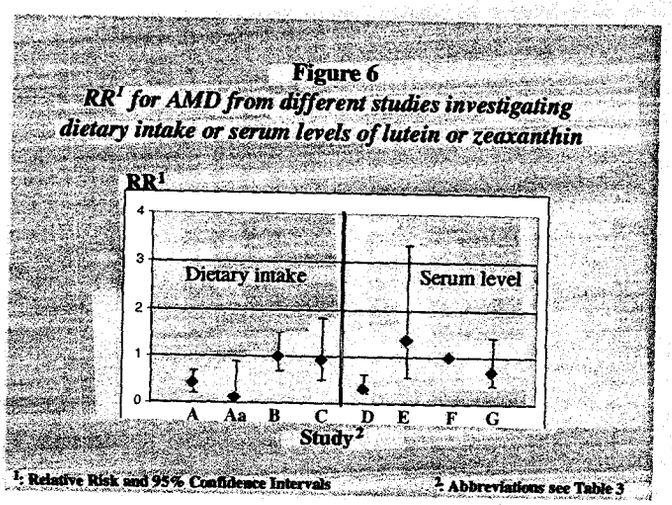


Figure 6
RR[1] for AMD from different studies investigating dietary intake or serum levels of lutein or zeaxanthin

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). Therefore, it is possible that AMD at least partly contributes to the preferential loss of lutein and zeaxanthin in the macula.

Table 4

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.

2.7.2 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.

2.7.3 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 makes 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.

2.8 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 2). 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.

2.9 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.

2.10 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.

2.11 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 chinense*, Gou Zi Qi (Table 2), 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.

2.12 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 5, the estimated dietary intake ratio of lutein to zeaxanthin from the whole diet varies roughly between 4:1 and 6:1 across age groups.

Table 5: 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 6) would be similar in aggregate to that reported by ENVIRON (although absolute intake estimates among population subgroups could vary widely).

Table 6: 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 6) and an estimated average dietary intake ratio of lutein : zeaxanthin around 5:1 (Table 5), 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 5). 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.

2.13 Conclusion

On the basis of the scientific ideas presented in this review, zeaxanthin and lutein would appear to have the potential to significantly contribute to a reduced risk of age-related macular degeneration, though in a way that cannot be precisely quantified as yet. Notwithstanding results of further experimental, epidemiological, and, most importantly, well-controlled clinical intervention trials, lutein and zeaxanthin appear to be valid candidates as ingredients for food intended to maintain retinal function.

To differentiate the detailed contribution of lutein and zeaxanthin is difficult, because only a small number of experiments have been carried out with pure 3R,3'R zeaxanthin and most experiments were done with lutein that contains approximately 5% 3R,3'R-zeaxanthin. To date, there is not sufficient evidence to determine if one is more efficacious or functionally important than the other. Based on chemical properties, zeaxanthin could be slightly more effective than lutein at protecting membranes against oxidative damage. Further research is needed to determine what levels of lutein and zeaxanthin intake best accommodate relevant physiological processes and to determine the potential of lutein and/or zeaxanthin to individually protect the eye against light and oxidative insult.

2.14 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 3

Stability and use of zeaxanthin

3.1 Stability

The beadlet formulation, 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 initial data after 3 months shows 100% stability at temperatures up to 35°C. We recommend that the product may be stored at a temperature below 15°C for up to 18 months.

3.2 Recommended use levels

Overall, there are few data available to serve as a basis for recommending levels of zeaxanthin 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 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 recipe database (ENVIRON, 1999), the CSFII 1994-96 food intake survey and the 1998 USDA Carotenoid Composition of Foods database. As shown in Table 5, the estimated ratio of lutein to zeaxanthin in the whole diet varies roughly between 4:1 and 6:1 across age groups.

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 6) would be similar in aggregate to that reported by ENVIRON (although absolute intake estimates among population subgroups could vary widely).

With a putative recommended intake of 6 mg/d (or higher) lutein + zeaxanthin based on observational studies (Table 6) and an estimated average dietary intake ratio of lutein : zeaxanthin around 5:1 (Table 5), 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 5). 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.

3.3 References

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Chapter 4

Toxicology summary

Zeaxanthin

Preclinical safety assessment

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4.1 Toxicology

4.1.1 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 had no genotoxic potential.

4.1.1.1 Ames tests

Mutagenicity evaluation of zeaxanthin, [redacted] in the Salmonella / microsome assay (Ames test). [redacted]

The [redacted] formulation 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 [redacted] 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 [redacted] zeaxanthin, thus protecting further for occurrence of mutagenic activity [redacted] zeaxanthin. [redacted]

The formulation for 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.

4.1.1.2 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 1 µg to 16 µg/ml (0.002–0.03 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)

4.1.1.3 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] er ml did not induce DNA repair synthesis in primary cultures of rat hepatocytes. (4)

4.1.1.4 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)

4.1.1.5 *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] 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] zeaxanthin /kg body weight. (6)

4.1.2 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)

4.1.3 Subchronic toxicity

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).

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)

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) of [redacted] was > [redacted] mg/kg in rats. (13)

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)

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 [redacted] 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)

4.1.4 Reproduction Toxicity

The teratogenicity of zeaxanthin was studied in rats and rabbits.

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).

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 rapeseed oil 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.

4.1.5 Chronic Toxicity

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

A one year tolerance study was performed in Cynomolgus monkeys to evaluate the toxicity of zeaxanthin in a [redacted] form by gavage administration at doses of [redacted] mg/kg/day to groups of 2 male and 2 female Cynomolgus monkeys. One additional male and female animal at [redacted] mg/kg/day were sacrificed after 6 month of treatment.

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.

Ophthalmoscopic findings included glittering deposits or spots and increased translucence that were observed in some animals in the ophthalmoscopic examinations after weeks 13, 26 and 52. During contact lens examinations, yellowish, plaque-shaped deposits and increased translucence was observed in control animals (week 15) and yellowish, gold-like and glittering deposits as well as punctiform and plaque-shaped deposits and whitish spots were observed in animals of groups 2 to 4 after weeks 15 to 18 and 26. These findings were not considered to be treatment-related. There were no treatment-related changes in electroretinograms.

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 (18).

The preliminary expert microscopic examination of the retina from the monkeys did not differentiate treated from control animals. An expert report on these examinations is in progress (19).

4.1.6 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. (20)

4.2 Absorption, Distribution, Metabolism and Excretion (ADME)

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] % in feed) 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 GI-tract. After 1 week, less than 0.5 % was still present in the body and less than 1 % 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. (21)

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. (22)

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

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

studies with astaxanthin and canthaxanthin. Absorption (biliary excretion not considered) varied from around [redacted] to around [redacted] (21, 23).

4.3 Conclusions

The safety of zeaxanthin was investigated by an array of studies, described in this document and listed in the Attachment.

The safety package adequately describes the safety of zeaxanthin.

There are no toxicity findings, which prevent the marketing of zeaxanthin 5% TG, from the safety point of view. With respect to the results of the subchronic toxicity studies of zeaxanthin, the product "can be regarded as safe". The only changes observed were treatment-related discolorations of fat and feces.

The toxicity testing of zeaxanthin was done 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 acute toxicity studies were done 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. Therefore, additional acute toxicity studies would be repetitions that are not allowed by ethical principles and national laws.

ADME studies revealed that zeaxanthin was rapidly leaving the body and GI-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.

4.4 References

1 [redacted] Mutagenicity evaluation of zeaxanthin, [redacted] in the Salmonella / microsome assay (Ames test). [redacted]

2 [redacted] Internal Communication: Decay of [redacted]-zeaxanthin. [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 [redacted]) administered orally as a feed admixture to mice over 13 weeks. [redacted]

13 [redacted] Tolerance study with [redacted] (zeaxanthin [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 study in rabbits with oral administration of [redacted] zeaxanthin. Segment II-teratological study. [redacted]

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

19 [redacted] Research Report in preparation

20 [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]

21 [redacted] Zeaxanthin Balance Studies. [redacted]

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

23 [redacted] Radioactivity in Expired Air during zeaxanthin Balance Studies compared to Previous Findings for canthaxanthin and astaxanthin with Rats. [redacted]

Attachment 1: Compilation of 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]	0, 44.5, 89.0, 178.0 mg/kg p.o.	Yes	1980	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]	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]	X	X	X	X	X ^{14}C
[redacted]	X	X	X	X	-
[redacted]	X	X	X	X	-
[redacted]	X	-	-	X	-
[redacted]	X	X	X	X	-
[redacted]	-	X	-	-	X
[redacted]	X	X	-	X	-
[redacted]	X	-	X	X	-
[redacted]	X	-	-	-	-
[redacted]	-	X	-	-	-
[redacted]	-	-	-	-	X
[redacted]	-	-	X	-	-

*In some mutagenicity studies (references # 1, 3, 4, 5) the material Ro 01-9509/007 denotes the crystalline form.

Chapter 5

Safety conclusions

5. Safety conclusions

The preclinical safety of zeaxanthin has been determined by 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. The subchronic toxicity studies show that the zeaxanthin product can be reasonably expected to be safe.

The average daily intake of zeaxanthin is considered to be approximately 170-190µg/d in the US, as calculated from CSFII 1994-1996 intake data and the 1998 USDA Carotenoid Database. The subchronic toxicity studies with supplementation of zeaxanthin in mice and rats resulted in a NOAEL of > 1000mg/kg bw/day and in dogs of >442mg/kg bw/day. A chronic toxicity study was carried out with monkeys, which were fed with doses of zeaxanthin up to 20mg/kg bw/day. No treatment-related effects were seen. Because there were no adverse effects in monkeys treated with zeaxanthin at a level of 20mg/kg bw/day, a level of 1200 mg/day of zeaxanthin is considered to be the no adverse effect level for humans (60 kg bw).

Natural food sources contain lutein and zeaxanthin in a ratio of approx. 5:1. Based on epidemiological studies it is estimated that the recommendation for average daily intake of the two carotenoids in combination could be at least 6 mg/day. Thus, the recommended average daily intake of zeaxanthin is considered to be 1 mg/day to help maintain healthy eyesight.

Based on the safety studies and human epidemiological data, a recommended use level of zeaxanthin as supplementation of 1 mg/day is considered to be safe.