Lutein and Zeaxanthin Measured Separately in the Living Human Retina with Fundus Reflectometry

Jan van de Kraats, Martijn J. Kanis, Stijn W. Genders, and Dirk van Norren

Purpose. To separately measure the optical densities of lutein (L) and zeaxanthin (Z) in the human retina in vivo. L and Z are the basic constituents of the macular pigment (MP).

Methods. Spectral fundus reflectance was measured in 23 subjects (group 1) at 0°, 1°, 2°, 4°, and 8° eccentricity with a modified macular pigment reflectometer. A model generated the optical densities of L (LOD) and Z (ZOD), using their slightly different absorption spectra. Three other subjects (group 2) took 20 mg/d zeaxanthin for 6 months; they were measured approximately monthly for 18 months.

Results. Mean LOD for group 1 at the central fovea was 0.200 ± 0.061 (range, 0.085–0.305), mean ZOD was 0.494 ± 0.169 (range, 0.169–0.806), resulting in a mean Z fraction [ZOD/(LOD + ZOD)] of 0.71. ZOD dropped faster toward the periphery than LOD, measuring 0.044 and 0.010 (Z fraction 0.18) at 8°, respectively. Zeaxanthin supplementation in group 2 caused a significant increase in ZOD, and no or minor changes in LOD. ZOD further increased over a 10-month period after supplementation in all subjects.

Conclusions. LOD and ZOD had different spatial profiles that, apart from scaling factors, showed similarity to in vitro literature data. Supplementation with Z caused LOD to decrease and ZOD to increase. These results strongly suggest that the optical densities of L and Z can be assessed in vivo by fundus reflectometry, opening new ways of investigating the putative protective roles of L and Z in retinal disease. (*Invest Ophthalmol Vis Sci.* 2008;49:5568–5573) DOI:10.1167/iovs.08-1939

Macular pigment (MP) has been hypothesized to play a role in protecting the retina against chronic light damage.[1–3] In the central fovea the highest concentration of macular pigment was found in the cone axons, but also some was seen in or near the cone outer segments. Just outside the foveola, the inner plexiform layer also contained high densities.[4,5] The macular pigment consists of both the isomeric carotenoids lutein (L) and zeaxanthin (Z), their concentrations peaking in the central fovea, with Z peaking more than L.[4,6,7] Mesozeaxanthin because of its similar absorption spectrum is often considered a part of Z. Z was associated by Bone et al.[6] with cone photoreceptors, while L was more associated with rod photoreceptors. Later Z as well as L was found in rod outer segments, and L was shown to be present in the central fovea devoid of rods.[7–9] Z is developed during the first 2 years of life.[6] Multimodal distributions have not been linked yet to Z or L.[4,10,11]

The Z component seems to be the most efficient in the quenching of singlet oxygen.[12,13] Also, the optical attenuation by Z extends to longer wavelengths.[14] Supplementation with foods rich in L and Z raise the density of macular pigment.[15–20] However, there are still many gaps in the knowledge on the pathways of uptake and metabolism of L and Z.[21] One difficulty is that until now, independent estimates of L and Z could be obtained in the retina only in vitro. In a recent study, for example, 32 monkeys were killed to obtain pertinent data.[16] It would be of great advantage if Z and L could be assessed in vivo. In principle, this can be achieved by taking advantage of the (small) differences in spectral absorption of L and Z.[14] The two absorption spectra are roughly similar in shape, but the spectrum of Z is shifted approximately 10 nm toward the longer wavelengths compared with the L spectrum. This shift is expected to have a small imprint on the spectral reflectance of the fundus, which is known also to show the fingerprints of other ocular pigments. Given sufficient wavelength resolution, the optical densities of these pigments can be estimated with an optical model of the fundus reflection.[22–25] Usually, these models have a free parameter for the optical density of macular pigment. In principle, this can be replaced by independent parameters for L and Z. The challenge with such optical reflection models lies in the validation of the output parameters. Avoiding in vitro experiments, we devised two experiments for that purpose.

First, L and Z were measured as a function of eccentricity. L and Z have, according to the in vitro literature, different spatial density profiles, both dropping off rapidly toward the peripheral retina, with Z declining faster than L.[6,14,26] In terms of the Z fraction, values of approximately 0.7 are found in the fovea, dropping to approximately 0.6 at 4° and to approximately 0.3 above 20° eccentricity.

Second, we supplemented three subjects with a daily dose of pure Z for 6 months, expecting different effects on the optical densities of L and Z.[16]

Methods

Measuring LOD and ZOD

LOD and ZOD were assessed with the macular pigment reflectometer (MPR),[27] an instrument designed to measure the reflection of a 1° spot projected on the fovea from 400 to 880 nm. The spectral resolution is 5.8 nm, as provided by the manufacturer of the fiber spectrometer used (model USB2000; Ocean Optics, Inc. Dunedin, FL). Although not necessary for the MPR measurements, the pupil of the right eye in all subjects was dilated by topically instilling tropicamide 0.5% eye drops before the MPR measurements were performed, because of additional experiments with another apparatus, not reported here. The original MPR was modified to enable eccentric fixation by adding four small holes covered with a red filter to the retinal field diaphragm in the illumination pathway. The extra fixation targets appeared simultaneously; the subjects were asked to fixate the *n*th spot from the left. The data from the MPR were analyzed with a revised edition of an optical model that has, among others, new data on the absorption of the ocular media.[25,28] Briefly, the model contains a number of reflect-

From the Department of Ophthalmology, University Medical Center Utrecht, Utrecht, The Netherlands.

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Corresponding author: Jan van de Kraats, UMC Utrecht, Department of Ophthalmology, AZU E03.136, PO Box 85500, NL-3508 GA Utrecht, The Netherlands; jkraats@umcutrecht.nl.

ing and absorbing layers. It has three reflecting layers, one at the inner limiting membrane (ILM), one for the combined Fresnel reflections of the individual segments of the cone photoreceptor outer segment and from the retinal pigment epithelium (RPE), and one for the reflection from the choroidal space. Absorption anterior to the receptor layer takes place in the lens and in the macular pigment. Absorption posterior to the receptor layer is due to melanin and blood. Because of the high-measuring light levels, visual pigment was considered to be fully bleached; its absorption could therefore be ignored. The Levenberg-Marquardt routine[29] was used to fit the data with the model by minimizing χ^2 values. Parameters for the absorbing and reflecting layers were allowed to vary simultaneously, or they were fixed to a (stated) value. The model has an extinction spectrum of MP that is the sum of a fixed ratio of 0.3 L and 0.7 Z (Fig. 1), using L and Z extinction spectra according to Handelman et al.[14] For the present purpose, the free parameters LOD and ZOD were used, and MP optical density (MPOD) was equal to the sum of LOD and ZOD. Changes in parameters like melanin and blood with eccentricity have little or no influence on the macular pigment estimates because of their very different spectral absorption. For the present purpose, only LOD, ZOD, and the Z fraction [ZOD/(LOD+ZOD)] are discussed.

Subjects

The experiments were approved by the medical ethics committee of the UMC Utrecht and were conducted. Before the experiment started, the nature and possible consequences of the experiment were explained to the subjects and written informed consent was obtained according to the Declaration of Helsinki. In all subjects, the measurements were performed only in the right eye, at the temporal side of the retina. The investigator got feedback on fixation and blinks: If the last measured spectrum (continuously displayed at 1-second intervals) was very different from the previous one, the screen displayed high standard deviations. The investigator awaited a "quiet" period with low standard deviations to record a measurement. Optical reflection spectra were obtained in 23 young subjects (5 men, 18 women) aged 24.3 ± 2.7 years (group 1) at eccentricities 0°, 1°, 2°, 4°, and 8°. At each retinal location, corresponding to the different fixation targets, five spectra were measured, each optimized for maximum reflection around 550 nm. LODs and ZODs present the mean of the five estimates from the optical modeling. The measurements of this group were analyzed with their spectral media losses fixed at a value corresponding with the mean group age, except for the scattering losses of the Rayleigh type, which were set at 0, as seems appropriate for reflection measurements originating mainly from the layers posterior to the receptors.[25,28]

A second group (group 2) of three male subjects, not included in group 1, aged 28 (S1), 57 (S2), and 64 years (S3) at the start of the experiment, ingested 20 mg Z (Zeavision, St. Louis, MO) per day during 6 months. Measurements started 1 month before intake and continued for up to 10 months after the intake of Z was stopped. The measurements from these subjects were analyzed with their spectral media losses fixed according to their age, except for the Rayleigh losses which were set at 0 again.[28]

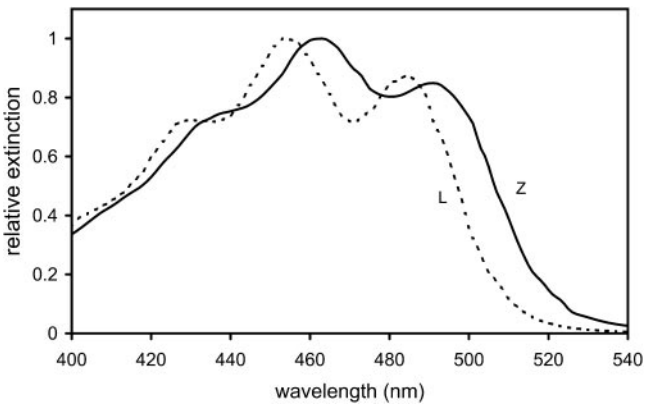

FIGURE 1. Relative extinction coefficient of L and Z.[14] The parallel slopes of the extinction spectra at the longer wavelengths show a shift of approximately 10 nm.

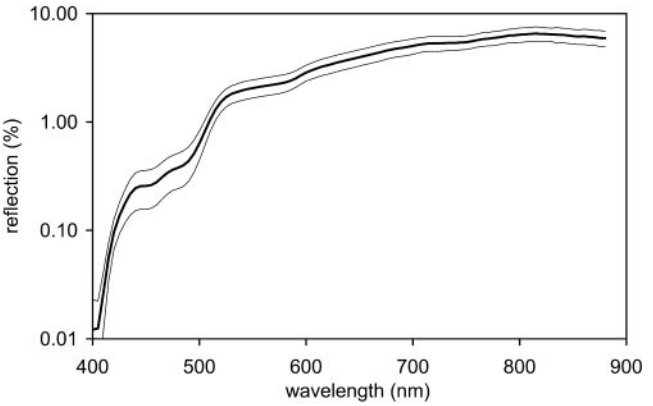

FIGURE 2. *Bold curve*: mean reflection spectrum from the central fovea of group 1. *Thin curves*: ±SD.

Statistical Analysis

Correlation between L and Z in group 1 was calculated with commercial software (SPSS ver. 11; SPSS Inc., Chicago, IL). Linear regression lines were drawn through the time traces of LOD and ZOD in group 2 (Excel 2003; Microsoft, Redmond, WA). Significance was calculated with the LINEST and FDIST functions. The level of significance was chosen as 0.05. To calculate the total change during and after the Z intake from the summation of the trend line results as shown in the eccentricity plots of Figure 5, the original standard errors were first combined in the root mean square way. If twice the resulting SE was smaller than the difference in the means, the before and after intake results were assumed significantly different.

RESULTS

To illustrate the outcome of an MPR measurement, Figure 2 shows the mean reflection spectrum from the central fovea in group 1. The decrease in reflection below 500 nm due to the absorption in L and Z is clearly visible. Below 420 nm, a further decrease is caused by the absorption in the eye lens. Above 580 nm the absorption in blood decreases rapidly, resulting in a higher reflection of light mainly originating from the choroid.[24,25] Finally, a slight decrease in reflection is seen at the longest wavelengths due to the absorption of water.

LOD and ZOD as a Function of Eccentricity

The mean retinal distribution of LOD and ZOD for the group of 23 young subjects (group 1) is given in Table 1. In Figure 3 the

TABLE 1. Retinal Distribution of LOD and ZOD in Group 1

Eccentricity (deg)	MPOD	LOD	ZOD	Z Fraction
Central	0.694 (6)	0.200 (24)	0.494 (8)	0.71
1	0.550 (8)	0.182 (23)	0.368 (9)	0.67
2	0.204 (13)	0.095 (24)	0.109 (13)	0.53
4	0.086 (14)	0.060 (30)	0.026 (29)	0.30
8	0.054 (35)	0.044 (46)	0.010 (73)	0.18

Data in parentheses are the coefficients of variation in percent (100 × SD/mean) calculated from the five measurements per subject

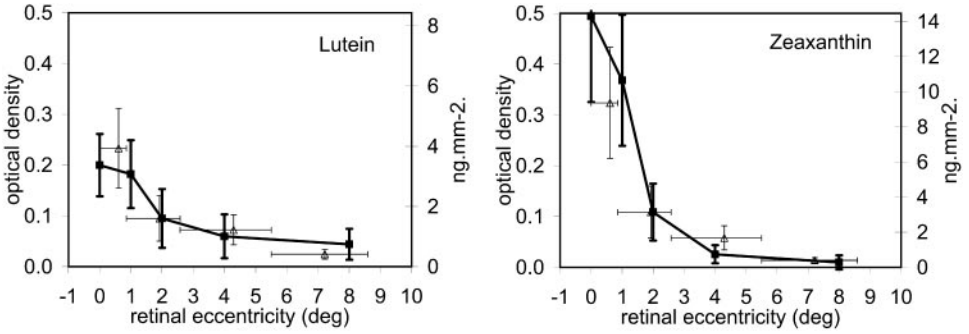

FIGURE 3. Optical densities as function of eccentricity for group 1 (■) of L (*left*), and Z (*right*). Error bar, SD. Also plotted are HPLC mass data for L and Z[6] (△, *right hand scale*), scaled to match the reflectometry data at 2° eccentricity. *Horizontal error bars*: retinal eccentricities from which the data were retrieved. Error bars, SD.

data are plotted together with the spatial distributions as found by Bone et al.[6] in human donor tissue with high performance liquid chromatography (HPLC). The latter data were scaled to fit our reflectometry data. Our range for LOD at the central fovea was 0.085 to 0.305; the range for ZOD was 0.169 to 0.806. LOD and ZOD showed no linear relationship (P = 0.485). Adding LOD and ZOD from each subject to obtain MPOD resulted in a mean of 0.694, with an SD of 0.171 and a range of 0.307 to 0.996. LOD and ZOD at the center of the fovea in both male and female subjects were not significantly different in a t-test (LOD, P = 0.81; ZOD, P = 0.73).

Z Supplementation

The effect of Z supplementation on LOD and ZOD for three subjects (group 2) at the central fovea is shown in Figure 4. During supplementation, only the LOD trend line of subject S1 showed a significant decrease in time (slope, −0.000256 (P = 0.0056). Of the LOD trend lines after supplementation, those of subject S1 (slope −0.000280 OD/d (P = 0.0008), and S2 (slope 0.000056 OD/d (P = 0.028) showed a significant change in time.

During supplementation, the time-dependent change in ZOD appeared significantly different from 0 in all subjects (S1, S2, and S3; slopes, respectively, 0.000834 [$P < 0.0001$], 0.000197 [$P = 0.013$], and 0.000537 OD/d [$P < 0.0001$]). After supplementation, regression analysis trend lines for ZOD were again significantly different from 0 in all subjects, with S1 having a slope of 0.000556 (P = 0.0004), S2 of 0.000249 ($P <$ 0.0001), and S3 of 0.000172 OD/d (P = 0.0023).

The starting point of a trend line during supplementation was used as a model estimate of the value before supplementation. The end point of the trend line for the period after

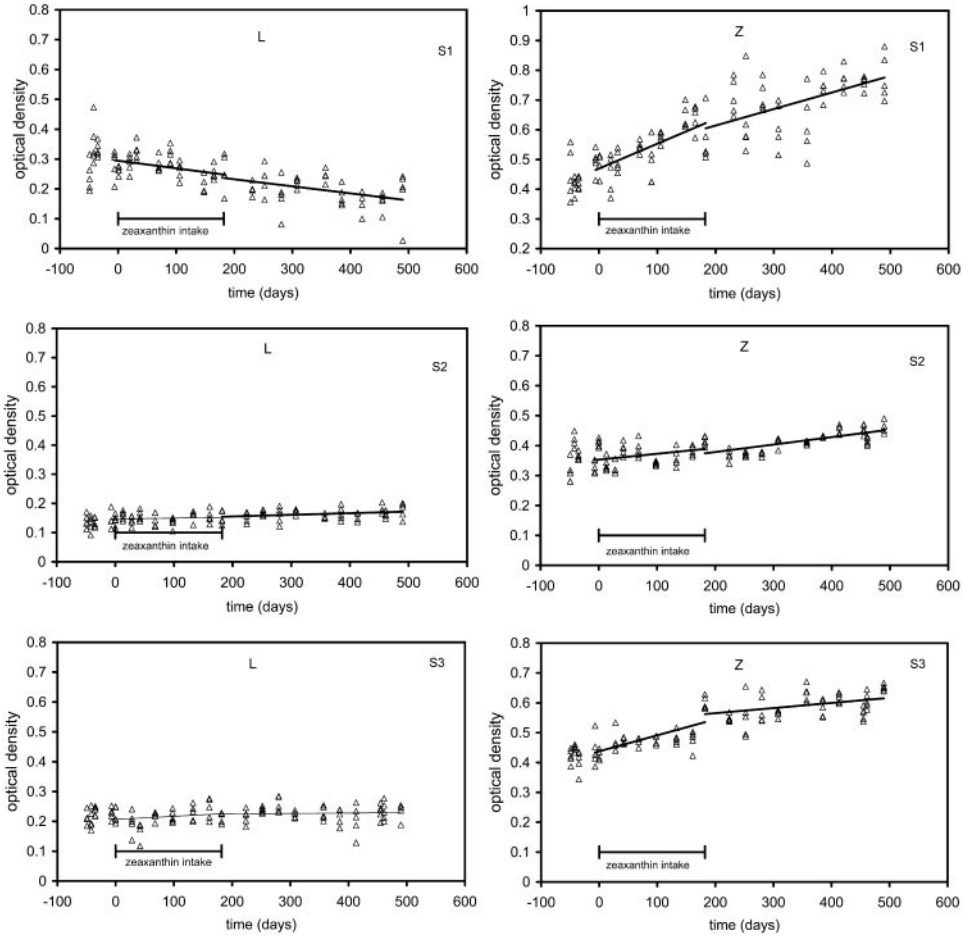

FIGURE 4. The optical density at the central fovea of L (*left*), and Z (*right*) for the three subjects. At each day of measuring, five measuring points are shown for each subject (some overlapping). From days 0 to 182 the subjects took 20 mg Z per day (*bar*). Trend lines during intake and during the period after intake are shown. *Bold trend lines*: significantly different from 0.

supplementation similarly provided a model estimate of the value approximately 300 days after termination of the supplementation. Figure 5 presents these estimates for ZOD and LOD before and after supplementation as a function of eccentricity. Three of five values of subject S1 showed a significant decrease in L; a few values of S2 and S3 were just significantly different, but also showed a decrease. All subjects showed an increase in Z at all eccentricities, apart from two eccentric positions in subject S1. A mean increase in MPOD of 0.109 (range, 0.058–0.161) in the central fovea was calculated, with no decline for up to 10 months after discontinuation.

Discussion

We modified the analysis software of the MPR,[27] incorporating the slightly different absorption spectra of L and Z, to obtain the optical densities of L and Z. The differences in spatial extent, combined with the different behavior of L and Z after supplementation of Z, strongly suggest that the method is capable of what it was designed to do. To our knowledge, this is the first time that ZOD and LOD were determined separately in vivo.

Technique

Successful analysis of LOD and ZOD depends critically on two factors: sufficient optical resolution and an adequate model analysis of the reflection spectrum. Spectral resolution was 5.8 nm, enough to distinguish between the approximately 10 nm difference in the long-wavelength flanks of L and Z. The optical model has proven its value in several studies on the macular pigment, in particular in a study in which different methods for measuring MPOD were compared, including heterochromatic flicker photometry (HFP) (Berendschot TT, et al. *IOVS* 2007; 48:ARVO E-Abstract 2138).

Although the optical model was developed for the cone rich central fovea, no limitation seems to exist for the analysis of ZOD and LOD at peripheral locations. The largest difference in retinal architecture between the central and more eccentric locations is that the nerve fiber layer becomes thicker with increasing eccentricity, thus leading to an increase in its reflectance.[30] The model adds this reflection to the reflection from the inner limiting membrane (ILM). A theoretical limitation of the MPR model, in contrast to that of the foveal reflection analyzer that takes into account the directional reflection of the cones (Zagers NPA, et al. *IOVS* 2001;42:ARVO Abstract 3779) is that it can only distinguish between the ILM and the retinal pigment epithelium (RPE) reflex when some amount of absorbing macular pigment is positioned between the two layers. Because in some cases the ILM reflection value exploded to unrealistically high values during the fitting process, we set its maximum limit to 0.32%, based on the sum of the mean and two standard errors in pilot experiments. A more detailed discussion of all the parameters generated by the optical model in the experiments is beyond the scope of this article. The circular measuring field of the MPR was 1°, which is expected to somewhat smooth the peaked distribution of macular pigment; this was not corrected for.

Would other techniques be capable of determining L and Z in vivo? The most wide-spread technique for measuring MPOD is heterochromatic flicker photometry.[31–33] With this technique, using a number of sufficiently stable test wavelengths with small enough bandwidth, it should in principle be possible. In terms of measuring speed, however, the (five times repeated) MPR measurement delivering a complete spectrum at a single eccentricity takes less than a minute, which is fast compared to the 15 minutes occasionally reported for heterochromatic flicker photometry for a single determination of MPOD only.[34] The present tech-

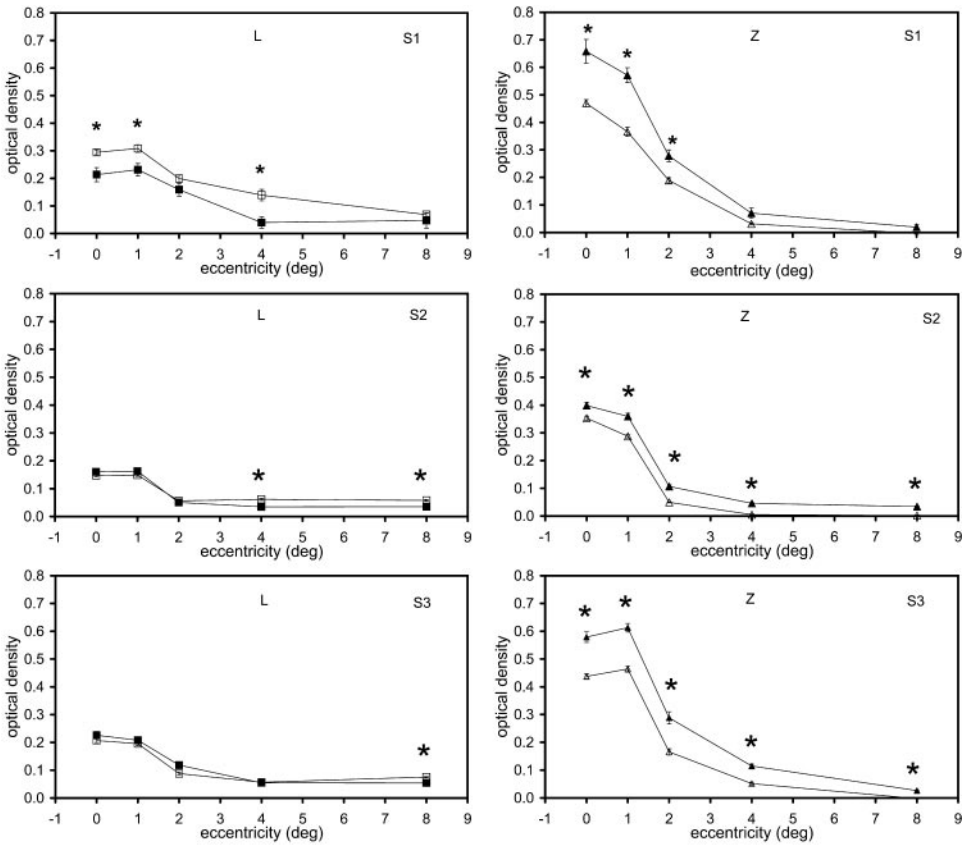

Figure 5. The mean optical densities for the three subjects of L (*left*) and Z (*right*) as a function of eccentricity before (*open symbols*) and after supplementation (*filled symbols*) as calculated from the trend-line modeling. Error bars, the calculated errors in the trend-line analysis. (*) significantly different levels.

nique also has no need for a reference location at 5° to 8° eccentricity. The Raman technique is not capable of distinguishing between L and Z, because their spectra are too similar.[35]

The mean result of 0.69 ± 0.17 for MPOD at the central fovea was higher than the previously reported 0.55 ± 0.21.[27] This is probably partly due to the changes in the template spectra used for the eye media and the macular pigments, and partly because of the large individual differences encountered between subjects.

ZOD and LOD as a Function of Eccentricity

The results for ZOD and LOD at different eccentricities obtained in 23 young subjects showed the general shape of the spatial distribution as found by Bone et al.[6] in human donor material, using HPLC. They found a relation between the distribution of Z and the cone distribution. In Figure 6 we plotted the Z fraction as a function of eccentricity and compared it to other in vitro data in human donor retinas.[6,8,26,36] This comparison serves only qualitative purposes. Plotting a fraction diminished the problem of differences in the sensitivity of the measurement techniques. However, the comparison remains riddled with uncertainties. L and Z, but in particular L, are arranged in macular tissue so as to absorb as much light as possible.[37,38] With HPLC, L and Z are dissolved in liquid and arranged randomly. In addition, HPLC takes into account only the membrane-bound L and Z, whereas at least part of the pigments are bound to a protein.[39–41]

Previous data have limited spatial resolution. In one study,[16] the data were even averaged over a macular region extending to almost 14°. Such results are dominated by the larger area at the most eccentric positions. Our data match well at low eccentricities, but higher eccentricities show a lower Z fraction. An explanation awaits a more straightforward comparison of in vivo followed by in vitro methods in animal experiments. Such experiments might also shed light on our finding of a significant optical density of MP at 8° eccentricity (0.054 ± 0.033). This issue is important because in HFP, this retinal position is often used as a reference, assuming MPOD to be 0 there.

We measured 5 men and 18 women in this study. For the sum of the macular pigments, as reported by Delori et al.,[10] the width of the retinal profile increases somewhat with age. This effect was larger in the women than in the men. Previously, Elsner et al.[42] found by SLO reflection that in subjects with older age (6 men, 5 women) alterations occurred like vanishing of the central peak, retaining a ringlike distribution. We found no significant differences between the L or Z distributions in men and women, but the low sample size of men seriously reduced the power. Future investigations may shed light on effects of age and sex in relation to the Z and L distribution.

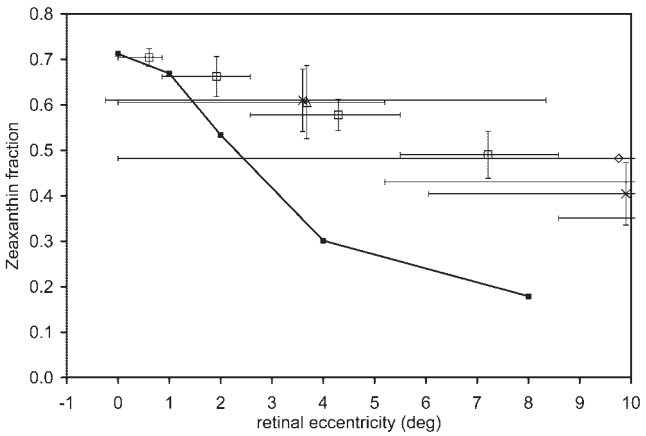

FIGURE 6. The Z fraction versus eccentricity (■) compared with human in vitro HPLC data from Bone et al.[6] (□), Handelman et al.[26] (◇), Bone et al.[36] (△), and Rapp[8] (×), these with an indication of the retinal areas over which they were derived.

Z Supplementation

In the past decade, several attempts have been made to increase MPOD by supplementation. This concerned certain foods and tablets containing pure L, pure Z, or a combination thereof, occasionally combined with additional supplements.[16,43] Depending on dose and duration of supplementation, an increase in the central fovea of approximately 0.1 density unit was found, with interindividual variations from 0 (nonresponders) up to 0.5 density units. The increase was seen to continue for a few months after discontinuation of the intervention, and no decline in MPOD was observed in two subjects up to 7 months.[44] Our findings with Z supplementation fit well into that picture with MPOD, showing a mean increase of 0.109 (range, 0.058–0.161) in the central fovea and no decline up to 10 months after discontinuation. Apparently, a modest period of intake can have a long-lasting (positive) effect on MPOD. To our knowledge, our follow-up period of 10 months is the longest hitherto reported.

Since in vivo detection of Z and L separately has not been described before, we have no human supplementation data to compare with ours. Supplementation with Z and L separately in rhesus monkeys was measured by Johnson et al.[16] using HPLC after the animals were euthanized. A quantitative comparison is impossible, because they analyzed over the whole central area of 16° in diameter. Second, the study started with animals on a diet free of Z or L, while supplementing the other ingredient. The message was that with supplementing only Z, this carotenoid was the only one to increase. For that reason, we selected Z supplementation in the present study. If we had supplemented only with L, it would probably have increased together with meso-Z, a substance with the same extinction spectrum as Z (and therefore included in our ZOD parameter). The conversion of meso-Z from L was suggested earlier by Bone et al.[36]

All subjects showed an increase of Z, the young subject (S1) the largest. Very young subjects are known to have a low Z content,[44] but this was not the case for the young adult in the present study. For L, the situation was less clear. Subjects S1 and S2 stayed close to their presupplementation levels, but the few significant changes involved decreases. Subject S1 decreased at three of five eccentricities. This effect is perhaps due to crowding with the increase in Z. Although the increase in Z occurred at all eccentricities, it was larger in an absolute sense at the central fovea, which had the largest values to begin with. In a relative sense, the increase in Z was largest at the more eccentric locations. This corroborates the finding of Johnson et al.[16] that high levels of supplementation have a large impact on peripheral locations. Clearly, a Z supplementation study with a substantial number of subjects is warranted to draw firmer conclusions.

CONCLUSION

We have described for the first time the in vivo and independent determination of the optical densities of Z and L as a function of retinal location by optical reflectometry. Z was most prominent in the central fovea, but dropped below the level of L at around 2° eccentricity, as found earlier with in vitro techniques. The relative ease of the new technique, together with its short measuring time, is promising in supple-

mentation studies. In retinal diseases, the independent detection of L and Z opens new ways for investigating their separate putative protective effects.

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