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Monthly Consistency of Macular Pigment Optical Density and Serum Concentrations of Lutein and Zeaxanthin

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ABSTRACT *Purpose*: This study was undertaken to assess serial month-to-month consistency of macular pigment (MP) optical density and serum concentrations of lutein (L) and zeaxanthin (Z). Four healthy subjects aged between 23 and 51 years volunteered to participate in this study. *Methods*: MP optical density (measured psychophysically using heterochromatic flicker photometry [HFP]), and serum concentrations of L and Z (quantified using high-performance liquid chromatography [HPLC]), were recorded every month for 24 consecutive months. *Results*: Mean MP optical density (±SD) was 0.361 (0.086) and 0.369 (0.074) for right and left eyes, respectively. There was no statistically significant seasonal variation in MP optical density for the group (two-way ANOVA: $p > 0.05$). Serum concentrations of L and Z demonstrated a statistically significant subject-season interaction effect (two-way ANOVA: $p < 0.01$). Serial serum concentrations of L and Z were positively correlated within all four subjects ($r = 0.370$ to 0.786), and significantly so for three subjects ($p < 0.05$). There was no obvious relationship, synchronous or lagged, between serum concentrations of L (or Z) and MP optical density ($r = -0.036$ to 0.368). *Conclusions*: MP optical density was relatively stable for all subjects over the 24-month period. Fluctuations in serum concentrations of L and Z, in the absence of dietary modification or supplementation, are associated with stable MP optical density.

KEYWORDS age-related macular degeneration; lutein; macular pigment; stability; temporal; zeaxanthin

INTRODUCTION

The macula is a specialized part of the retina that is responsible for central and color vision, which is initiated by the cones within this area. At the macula, there exists a yellow pigment known as macular pigment (MP), which is composed of two dietary carotenoids, lutein (L) and zeaxanthin (Z). It has been postulated that the absorptive and/or antioxidant properties of MP confer protection against development of various eye diseases such as age-related macular degeneration (AMD).[1]

In humans, MP cannot be synthesised *de novo* and is derived entirely from diet.[2] Several studies have investigated the relationship between dietary and serum concentrations of L (and Z), and MP optical density in humans, and all have demonstrated a positive relationship between these variables.[3–11] Furthermore, human studies have consistently shown a rise in MP optical density, in a majority of subjects at least, after dietary supplementation with L and Z.[4,12]

Various factors have been shown to be associated with serum L (and Z) and/or MP optical density, and these include: age,[9,13,14] gender,[15,16] body fat,[9,17] ultraviolet light exposure,[18] smoking and drinking habits,[9,19,20] time of day and season,[12,15,21,22] and geographic location.[23] However, there is a consensus that dietary intake of L and/or Z represents one of the most important determinants of MP optical density, consistent with cross-sectional and supplementation studies.[4,12,24]

Serum concentrations of carotenoids reflect recent dietary intake and may show a seasonal variation.[22] Such fluctuations may be attributable to season-dependent availability of various fruit and vegetables throughout the year. Moreover, the concentration of carotenoids also show a seasonal variation in certain food components.[25,26] To date, no study has investigated whether MP optical density fluctuates on a seasonal basis and whether such fluctuations (if any) relate to changes in serum concentrations of its constituent carotenoids. Hammond et al. have reported longitudinal data on MP optical density (central 1-degree) for 10 subjects over periods ranging from 1 to 16 years.[27] However, due to the unsystematic nature of the subject recruitment dates, it is difficult to draw any firm conclusions regarding the seasonal stability of MP from this study. Their findings do, however, suggest that MP optical density (for the 1-degree test) is reasonably stable over long time periods (1–16 years), with maximum and minimum ranges of 0.21 log units (9 month interval) and 0.03 log units (14 month interval), observed for MP optical density, respectively.

This study is designed to report serial monthly measurements of MP optical density and serum concentrations of L and Z in four healthy volunteers over a 24-month period and to investigate whether there is a relationship between any observed fluctuations of MP optical density and serum concentrations of L (and/or Z).

MATERIALS AND METHODS

Subjects

Four healthy subjects, two male and two female, aged between 23 and 51 years volunteered to participate in this study, which was approved by the Research Ethics Committee of the Waterford Institute of Technology and Waterford Regional Hospital. Informed consent was obtained from each subject, and the procedures adhered to the tenets of the Declaration of Helsinki.

All subjects were white, nonsmokers, with best-corrected visual acuity of 0.1 or better (logMAR). Also, all subjects lived in the southeast of Ireland, had normal eating habits, and none were on a special diet or taking carotenoid supplements. Their body mass index, percentage body fat (measured by dual energy x-ray absorptiometry),[9] serum cholesterol, and triglyceride concentrations all fell within normal ranges. Further, all subjects were experienced in psychophysical testing and were aware of the purpose of the study. MP optical density and serum levels of L and Z were obtained on the last week of every month over a 24-month period (January 2003 to December 2004).

Measurement of Macular Pigment

MP was measured psychophysically by means of a principle known as heterochromatic flicker photometry (HFP). We used a portable screening instrument, which has been described in more detail elsewhere.[28]

Principle

The concentration of MP peaks at the center of the fovea and is optically undetectable at an eccentricity of 6.5 degrees.[29] As MP absorbs blue light (peak absorption at 460 nm), and because of its anatomical distribution, the premise of matching two lights (one blue and one green) for equal luminance when their image is at the macula (foveal) and outside the macula (parafoveal) can be used to determine the central peak optical density of MP in a technique known as HFP.

The field that is imaged on the fovea is viewed at a distance of 330 mm and subtends a diameter of 1 degree at the eye. For the minimum-flicker match that is made away from the fovea, and where there is no MP, the test field is two arcs, concentric with the foveal field of 10 degrees inner diameter and of 1-degree width. Using the instrument, a green light of constant luminance flickers in square wave counter phase with a blue light, the

luminance of which can be varied by the subject, in order to achieve a state of minimum flicker (the matching luminance). The luminance of the plateau of the green square wave was known and that of the blue square wave was measured by inbuilt calibrated photodiodes. The logarithm ratio of the luminance of blue light required to achieve minimum flicker (log [foveal/parafoveal]) is directly related to the amount of pigment present and is known as MP optical density.

The subject may never observe a state of no flicker because with the instrument we used, there was no option to adjust flicker frequency. This is a limitation of the current instrument, as recent work has shown that the inability to adjust flicker frequency on an individual level may lead to increased uncertainty in achieving the match end-point, particularly in older people where the critical fusion frequency (CFF) reduces with age.[30] However, all our subjects were reasonably young (range, 23–51 years) and experienced users of Professor Mellerio's instrument.

For all subjects, the coefficient of variation (CoV) of the six blue luminance settings made in the fovea or the parafovea for each MP optical density measurement in any one session remained below 10%. It is part of the protocol when using this instrument that subjects in whom the COV exceeds 20% are excluded from the study. With minimum practice, the subjects were able to achieve matching luminance (minimum flicker) without difficulty.

Procedure

All subjects were experienced in psychophysical testing and had used the instrument on several occasions prior to commencement of this study. However, whenever a subject was tested, the investigator explained the principles of making a minimum-flicker match, in order to facilitate measurement.

The instrument was set up on a tabletop in a well-lit office, at an angle of about 35 degrees. Subjects were allowed to make two or three trial minimum flicker matches before recording of measurements commenced and then were encouraged to make the matches quickly. Perfectionist adjustment of the blue light luminance control was actively discouraged in order to avoid the well-known problems of adaptation and the Troxler effect (see, for example, Moreland et al.).[31] When the subject was satisfied that minimum flicker had been achieved, he or she pressed the sample and hold button, which measured the blue luminance. After each match was recorded, the investigator set the blue light luminance control to some new random position so that the subject did not learn how far to adjust the control to obtain a match. In each session, six foveal readings were obtained, followed by six parafoveal readings, for each eye. For a detailed description of the instrument (principle, apparatus, procedure), see previously published work by the Macular Pigment Research Group (MPRG). [9]

Serum Lutein and Zeaxanthin Analysis

Collection

Blood samples (6–8 ml) were collected from all subjects, on the same day as the MP optical density was measured. For investigation of possible diurnal variation, blood samples were taken at 0830, 1030, 1400, and 1800, from all subjects. Serum was separated from blood by centrifugation at 5000 rpm for 10 min and then aliquoted into 3 light sensitive microcentrifuge tubes and stored at -70°C until time of analysis.

Instrumentation

Serum L and Z were determined using reverse-phase, high-performance liquid chromatography (HPLC). We used a Hewlett-Packard (HP 1090 LC; Agilent, Dublin, Ireland) system with photodiode array detection at 295, 325, and 450 nm. A 5-μm analytical/preparative 4.6 $\times$ 250 mm 201TP speciality reverse phase column (Vydac, Hesperia, CA, USA) was used with an in-line guard column. The mobile phase consisted of 97% methanol and 3% tetrahydrofuran and was degassed using an in-line degasser. The flow rate was 1 ml/min, and the total run time was 15 min. All carotenoid peaks were integrated and quantified using Agilent Chem Station software.

Extraction

Duplicate extractions were carried out for each serum sample. A 0.4 ml aliquot of serum was pipetted into a light screening microcentrifuge tube (2 ml total capacity). Ethanol (0.30 ml) containing 0.25 g/L butylated hydroxytoluene (BHT) and internal standard (tocopherol acetate) was added to each tube. Heptane (0.5 ml) was then added and samples were vortexed vigorously for 1 min followed by centrifugation at 2000 rpm for 5 min (MSC Micro Centaur, Davison & Hardy Ltd., Belfast, UK). The resulting heptane layer was retained and transferred to a second labeled light-sensitive

TABLE 1 **Diurnal Variation of Macular Pigment Optical Density (MPOD), Serum Lutein, and Serum Zeaxanthin**

Subject	Mean MPOD right eye[a]	Range MPOD right eye	Mean serum L[b] (μg/ml)	Range serum L (μg/ml)	Mean serum Z[c] (μg/ml)	Range serum Z (μg/ml)
1	0.348 ± 0.020	0.334–0.362	0.101 ± 0.002	0.099–0.105	0.030 ± 0.002	0.0281–0.0326
2	0.451 ± 0.027	0.432–0.470	0.097 ± 0.005	0.089–0.099	0.031 ± 0.001	0.0306–0.0336
3	0.330 ± 0.028	0.310–0.349	0.063 ± 0.004	0.058–0.068	0.021 ± 0.001	0.0197–0.0222
4	0.377 ± 0.023	0.360–0.393	0.061 ± 0.005	0.056–0.066	0.024 ± 0.001	0.0255–0.0227

Subjects 1 and 2 male, subjects 3 and 4 female.
[a]Mean (±SD), MP optical density recorded during 2 different sessions.
[b]Mean (±SD), serum lutein recorded at 4 different time points.
[c]Mean (±SD), serum zeaxanthin recorded at 4 different time points.

microcentrifuge tube, and a second heptane extraction was performed. The combined heptane layers were immediately evaporated to dryness under nitrogen using a sample concentrator (Techne Sample Concentrator; Davison & Hardy Ltd). These dried samples were reconstituted in methanol (200 μl), and 150 μl was injected for HPLC analysis.

DSM Nutritional Products (Basel, Switzerland) provided L and Z standards, which were used to generate standard curves for quantification of serum concentrations of these carotenoids. This assay has been validated against the National Institute of Standards and Technology (NIST) Standard Reference Material 968c for Fat-Soluble Vitamins, Carotenoids and Cholesterol in Human Serum.

Statistical Analysis

The data was analyzed using the statistical software packages SPSS version 11 (SPSS, Chicago, IL, USA), and MINITAB version 13. Each volunteer's monthly series of MP optical density measurements and serum concentrations of L (and Z) were subjected to formal and informal statistical tests for outliers, stationarity (stability) of mean and variance, autocorrelation, and correlation between series. No outliers (defined as a value outside the interval mean ±3 ∗ standard deviation) were found for any of these data sets. Conventional analysis of variance (ANOVA) was used where appropriate, with season as a fixed factor and subject as a random factor. Residual analysis showed that the normality and independence assumptions needed for ANOVA were approximately satisfied.

RESULTS

The mean age (±SD) of the four subjects was 36.25 (11.47) years and ranged from 23 to 51 years. Of the four subjects, two were male and two were female.

MP Optical Density

Diurnal Variation

The means, standard deviations, and ranges of MP optical density measurements for all four subjects, taken at two different times in a single day (separated in time by at least 4 hrs), are shown in Table 1.

Seasonal Variation

The means, standard deviations, and ranges of MP optical density (right eye) measurements for all four subjects, taken from January 2003 to December 2004, are shown in Table 2. The monthly variation in terms of peaks and troughs is depicted graphically for all subjects in Figures 1–4. Changes in MP optical density correlated well for right and left eyes in subjects 2, 3, and 4, represented by r values of 0.704 ($p < 0.01$), 0.622 ($p < 0.01$), 0.433 ($p < 0.05$), respectively (Fig. 5). For subject 1, the variation observed in MP optical density between both eyes was asymmetrical ($r = 0.057$, $p > 0.05$) (Fig. 5). However, after exclusion of data pertaining to the 4 months where inter-ocular symmetry of MP optical was poorest for subject 1 (months 9, 19, 22, and 24), this correlation became significant ($r = 0.477$, $p < 0.05$, $n = 20$ months).

The group means and standard deviations of MP optical density measurements for the four subjects taken with respect to the seasons (summer = May, June, July; autumn = August, September, October; winter = November, December, January; spring = February, March, April) over a 2-year period are shown in Table 3. There was no statistically significant interaction between subject and season (two-way ANOVA: $p > 0.05$), and no statistically significant seasonal variation observed in MP optical density for the group (two-way ANOVA: $p > 0.05$) (Fig. 6). However, there was a statistically significant subject effect, independent of season,

TABLE 2 **Measurement of Macular Pigment Optical Density (MPOD) and Serum Lutein and Zeaxanthin Over a 24-Month Period (January 2003 to December 2004) for the Four Subjects**

Subject	MPOD right eye[a]	Range MPOD right eye	Serum L (μg/ml)[b]	Range serum L (μg/ml)	Serum Z (μg/ml)[c]	Range serum Z (μg/ml)
1	0.317 ± 0.040	0.231–0.371	0.093 ± 0.021	0.060–0.130	0.035 ± 0.009	0.020–0.054
2	0.474 ± 0.054	0.347–0.600	0.096 ± 0.026	0.059–0.161	0.037 ± 0.011	0.020–0.057
3	0.275 ± 0.049	0.189–0.366	0.067 ± 0.019	0.040–0.101	0.022 ± 0.005	0.010–0.032
4	0.379 ± 0.055	0.288–0.508	0.102 ± 0.023	0.056–0.141	0.032 ± 0.009	0.018–0.052

Subjects 1 and 2 male, subjects 3 and 4 female.
[a]Mean (±SD), MP optical density measured over a 24-month period (January 2003 to December 2004).
[b]Mean (±SD), serum L measured over a 24-month period (January 2003 to December 2004).
[c]Mean (±SD), serum Z measured over a 24-month period (January 2003 to December 2004).

with respect to MP optical density (two-way ANOVA: $p < 0.001$) (Fig. 7).

Serial Autocorrelation

No significant autocorrelations, at lags of 1, 2, or 3 months, were found for any subject ($p > 0.05$, for all) (Figs. 1–4).

Serum Lutein

Diurnal Variation

The means, standard deviations, and ranges of serum L measurements for all four subjects, taken at four different time points, on a single day, are shown in Table 1. There was no statistically significant diurnal variation observed for serum concentrations of L in any subject, as demonstrated graphically in Figure 8 (two-way ANOVA: $p > 0.05$).

Seasonal Variation

The means, standard deviations, and ranges of serum L measurements for all four subjects, taken from January 2003 to December 2004, are shown in Table 2. The monthly variation, in terms of peaks and troughs, is depicted graphically for all subjects in Figures 1–4.

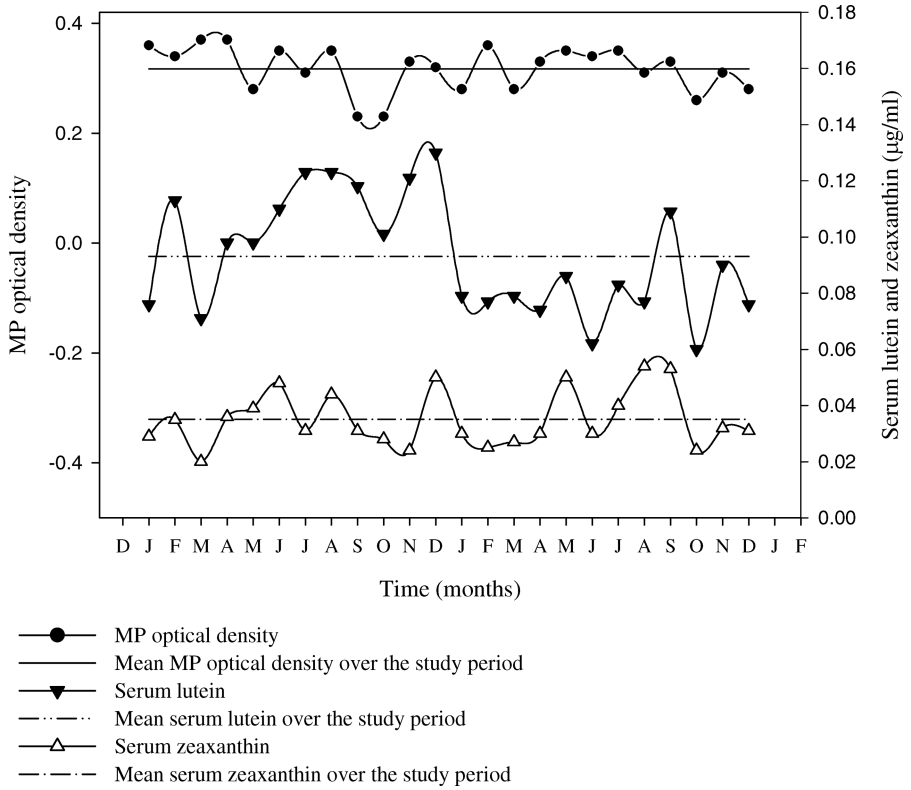

FIGURE 1 **Monthly MP optical density and serum concentrations of lutein and zeaxanthin measurements over a 24-month period (subject 1).**

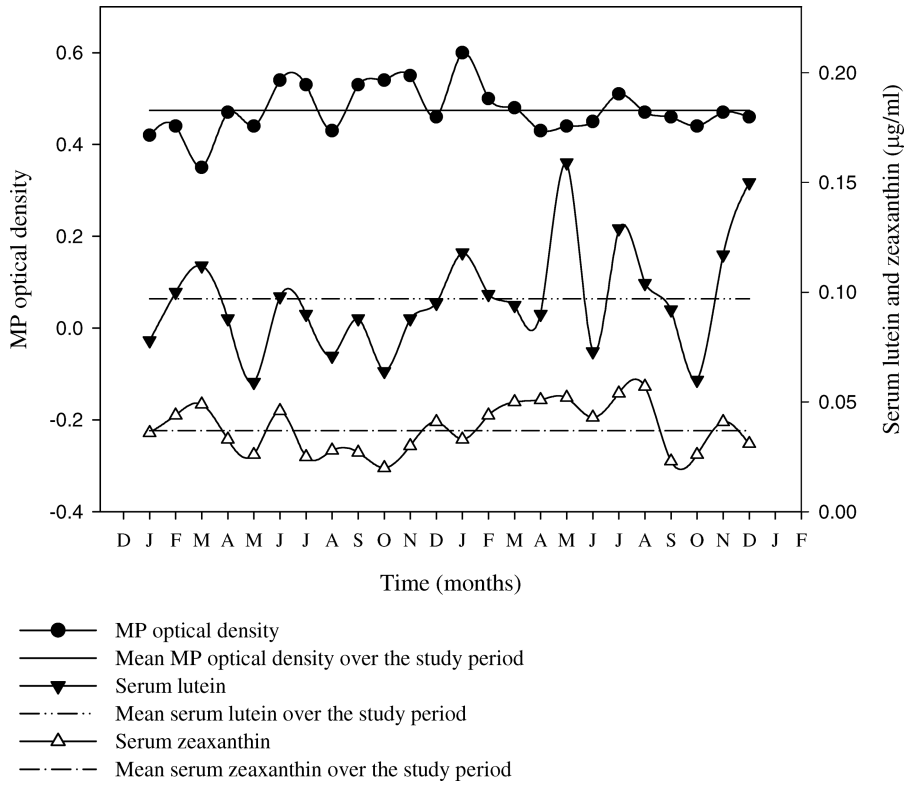

FIGURE 2 **Monthly MP optical density and serum concentrations of lutein and zeaxanthin measurements over a 24-month period (subject 2).**

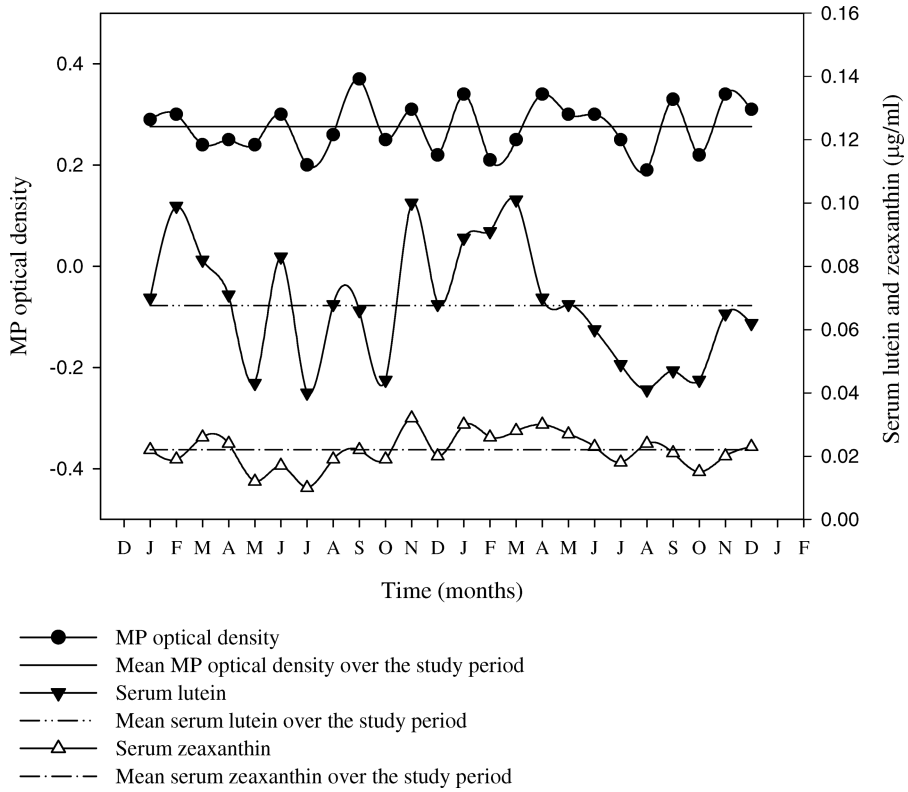

FIGURE 3 **Monthly MP optical density and serum concentrations of lutein and zeaxanthin measurements over a 24-month period (subject 3).**

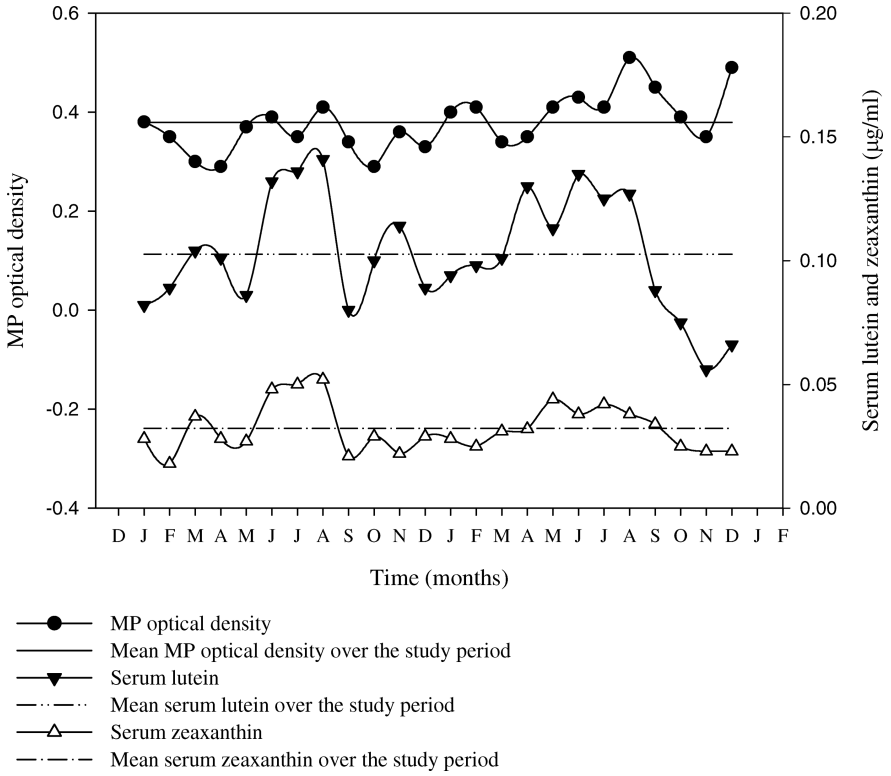

FIGURE 4 **MP optical density and serum concentrations of lutein and zeaxanthin measured every month over a 24-month period (subject 4).**

The group means and standard deviations of serum L measurements taken for the four subjects with respect to the seasons over a 2-year period are shown in Table 3. A significant subject-season interaction effect was found (two-way ANOVA: $p < 0.01$), and is illustrated graphically in Figure 9.

Serial Autocorrelation

The monthly series of serum L readings was tested for serial correlation within subjects. The results were rather multifaceted. For subject 1, the 2-month lag correlation ($r_2 = 0.466$, $p < 0.05$) was higher than the 1-month lag correlation ($r_1 = 0.316$, $p > 0.05$). Subject 3 demonstrated a similar, albeit insignificant, pattern ($r_2 = 0.387$, $r_1 = 0.239$; $p > 0.05$ for both). For subject 2, r_1 and r_2 were virtually 0, and for subject 4, r_1 and r_2 were 0.48 ($p < 0.05$) and 0.146 ($p > 0.05$), respectively.

Serum Zeaxanthin

Diurnal Variation

The means, standard deviations, and ranges of serum Z measurements for all four subjects, taken at four different time points on a single day, are shown in Table 1. There was no statistically significant diurnal variation observed for serum concentrations of Z in any subject, as demonstrated graphically in Figure 8 (two-way ANOVA: $p > 0.05$).

Seasonal Variation

The means, standard deviations, and ranges of serum Z measurements for all four subjects, taken from January 2003 to December 2004, are shown in Table 2. The monthly variation in terms of peaks and troughs is depicted graphically in Figures 1–4.

TABLE 3 **Macular Pigment (MP) Optical Density and Serum Levels of Lutein and Zeaxanthin with Respect to the Seasons During the Study Period (January 2003 to December 2004)**

	Summer	Autumn	Winter	Spring
Lutein (μg/ml)	0.093 ± 0.033	0.082 ± 0.028	0.090 ± 0.024	0.093 ± 0.014
Zeaxanthin (μg/ml)	0.035 ± 0.013	0.030 ± 0.012	0.029 ± 0.007	0.032 ± 0.009
MP optical density	0.368 ± 0.090	0.357 ± 0.104	0.373 ± 0.091	0.347 ± 0.077

n = 4 subjects over 24-month period.

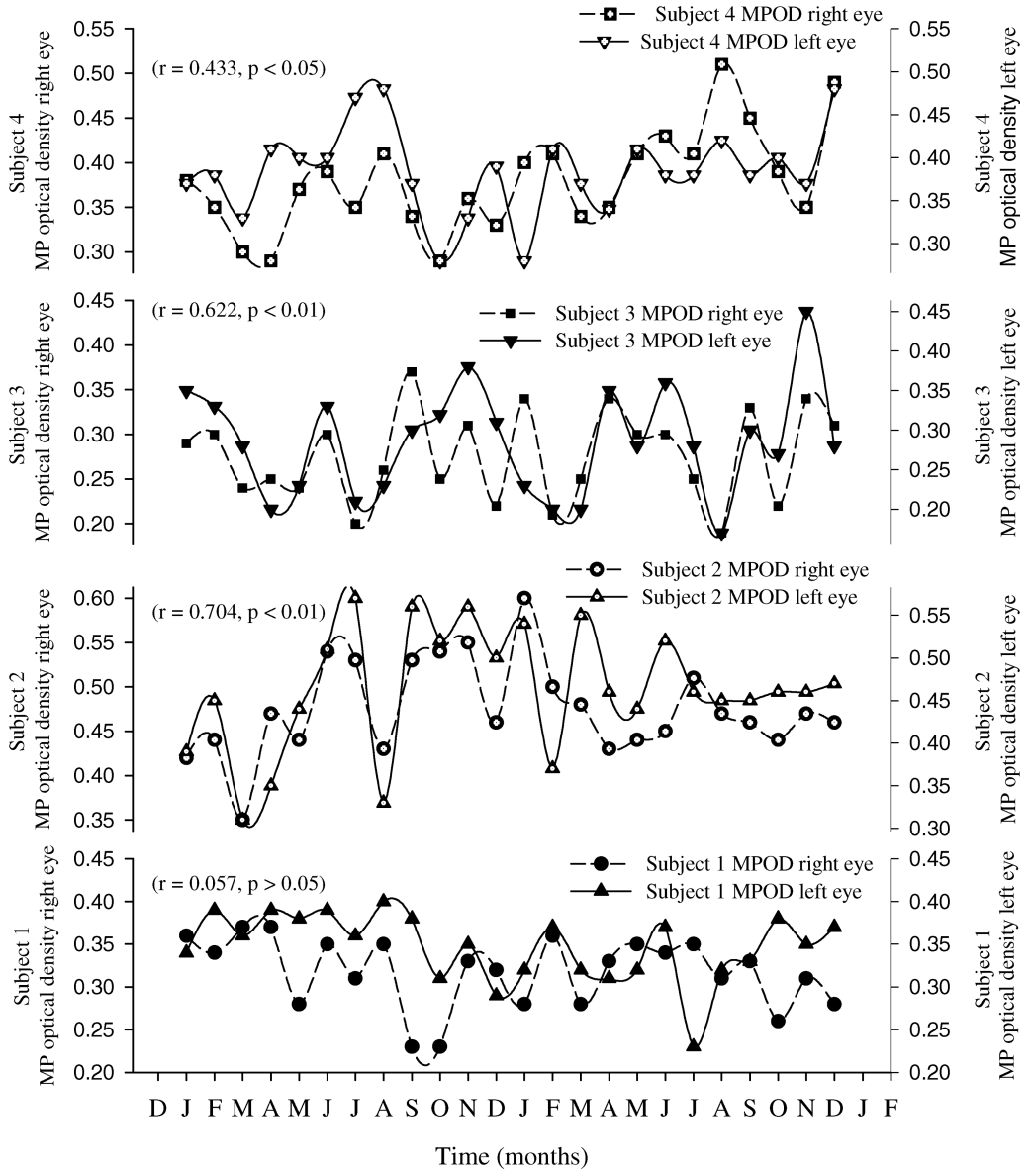

FIGURE 5 **Perennial variation of MP optical density for right and left eyes over 24-month period.**

The group means and standard deviations of serum Z measurements taken for the four subjects with respect to the seasons over a 2-year period are shown in Table 3. A significant subject-season interaction effect was found (two-way ANOVA: $p < 0.01$), and is illustrated graphically in Figure 9.

Serial Autocorrelation

The monthly series of serum Z readings was tested for serial correlation within subjects. One-month lag autocorrelations were significant in subjects 2 and 4, represented by r_1 values of 0.381 and 0.385, respectively ($p < 0.05$ for both). The 2-month lag autocorrelation was significant in subject 3 only ($r_2 = 0.358$; $p < 0.05$).

Relationships Between Serum Lutein, Zeaxanthin, and MP Optical Density

For all subjects, fluctuations in serum L were consistently accompanied by parallel changes in serum Z (subject 1: $r = 0.370$, $p = 0.075$; subject 2: $r = 0.490$, $p < 0.05$; subject 3: $r = 0.628$, $p < 0.01$; subject 4: $r = 0.786$, $p < 0.01$) (Figs. 1–4).

Figures 1–4 fail to demonstrate an obvious relationship, synchronous or lagged, between serum concentrations of L (or Z) and MP optical density. Correlations between monthly MP and monthly serum concentrations of L, for subjects 1–4, were −0.036, 0.079, 0.296, and 0.107, respectively, $p > 0.05$ in all cases (Figs. 1–4).

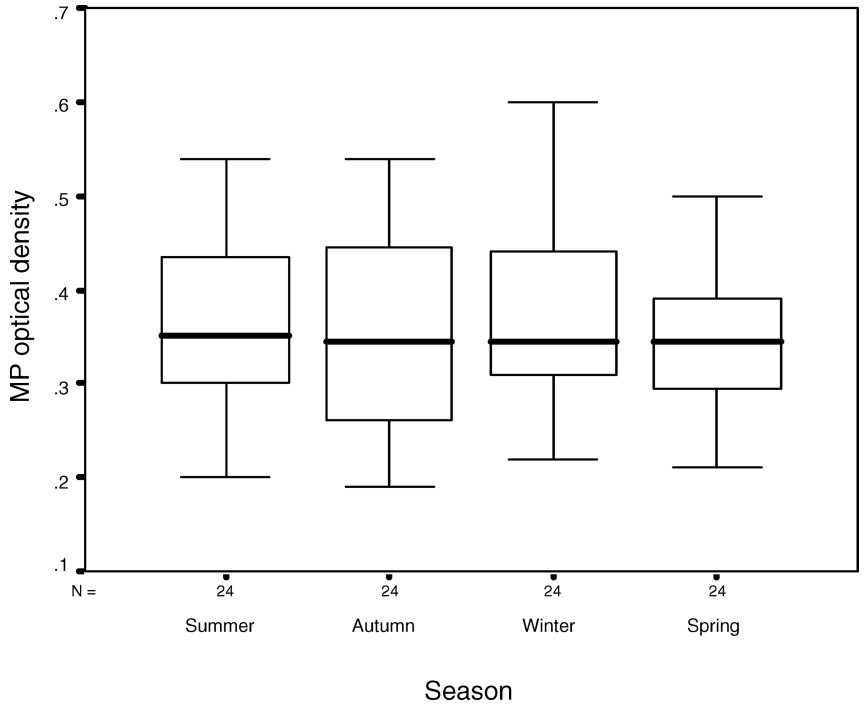

FIGURE 6 **Boxplot of group MP optical density with respect to season.**

Correlations between monthly MP and monthly serum concentrations of Z, for subjects 1–4, were 0.174, 0.266, 0.368, and 0.202 respectively, $p > 0.05$ in all cases (Figs. 1–4).

DISCUSSION

This study was carried out to examine serial monthly measurements of MP optical density, and serum concentrations of L and Z, in four healthy volunteers over a

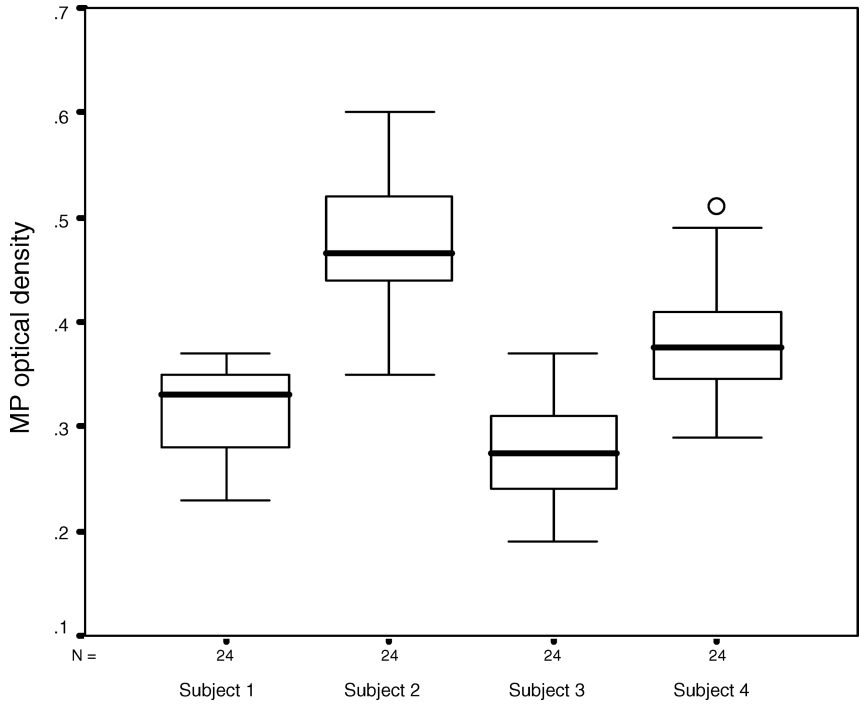

FIGURE 7 **Boxplot of MP optical density for individual subjects over the study period.**

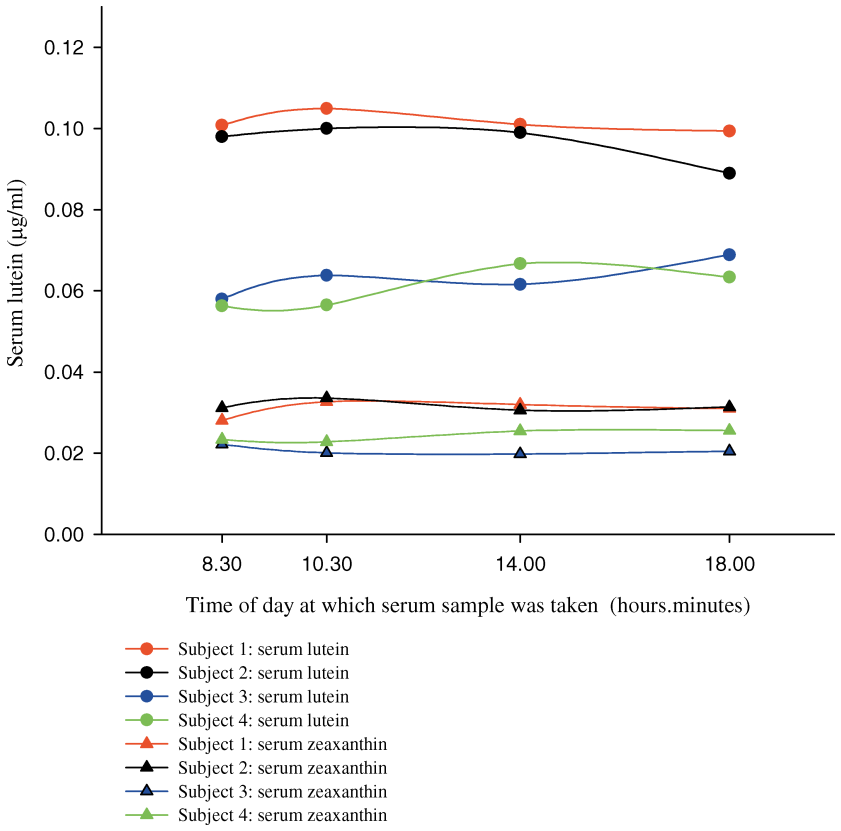

FIGURE 8 **Diurnal variation of serum lutein and zeaxanthin concentrations.**

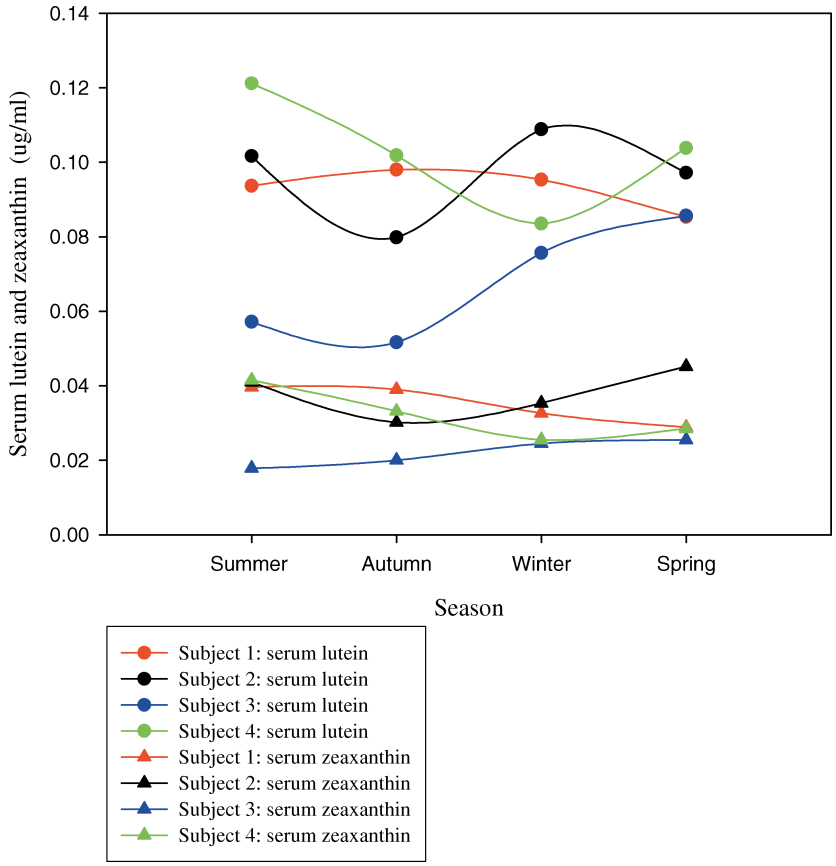

FIGURE 9 **Average seasonal concentration of serum lutein and zeaxanthin, by subject.**

24-month period. In addition, we investigated whether any observed fluctuations of MP optical density and serum concentrations of L (and/or Z) were related, and whether there was any evidence of seasonal variation in MP optical density and/or serum concentrations of its constituent carotenoids.

Mean MP optical density among our four subjects was 0.361, which is comparable to previous reports for populations of similar age groups and demographics.[9,32–36] In general, MP optical density remained stable over the study period, for all subjects. The time series of monthly MP optical density measurements were stationary in terms of the mean and variance, for three of the four subjects. However, there was a small, but statistically significant, increase in mean MP optical density for subject 4 over the 2-year study period.

Negligible differences between MP optical density measurements taken at two time points (separated by at least 4 hr) in a single day were observed and are represented by a mean difference of 0.01 (0.03) for the group. Examining the series of all four subjects, and using the diurnal ranges for MP optical density in Table 1 as indicators of measurement error, we can conclude that there is greater month-to-month variation in MP optical density than can be ascribed to measurement error. This finding is consistent with work carried out by Hammond et al. who reported significant daily differences in MP optical density for a given eye.[37] Changes in MP optical density over time, in the absence of dietary modification or supplementation, may be attributable to a variety of factors that influence the repletion/depletion balance of these carotenoids in the retina. Accumulation of the carotenoids at the macula depends on dietary intake,[38] absorption and transport of L and Z,[39] as well as (as yet unidentified) factors that influence retinal capture of these compounds.[40] It seems, therefore, that stabilization, and hence depletion, of MP may be influenced by the oxidant load in its local environment at any given time. In other words, there are many factors that are likely to influence MP levels and contribute to time-related fluctuations of this pigment.

For MP optical density, subjects 1 to 4 all exhibited considerable variation in their monthly measurements. For example, for subject 1, the maximum MP optical density value was 16.5% above the mean level for this subject, and the minimum was 27.6% below the mean level. The clinical importance of this variation remains elusive and therefore merits further study. However, statistically speaking, there were no outliers (i.e., at no point over the 24-month period did the MP optical density exceed the range mean $\pm$ 3 $*$ standard deviation for any subject).

Furthermore, there was no statistically significant season effect, or season-subject interaction, for MP optical density, whereas there was a statistically significant subject effect. This high degree of inter-subject variability in terms of MP has been reported elsewhere.[27]

We examined the monthly series of MP optical density measurements for autocorrelation. The autocorrelation coefficient, r_k, is a measure of the correlation between values, separated by k months, of a given subject's series. In brief, at the 5% level of significance, there was no evidence of serial autocorrelation in any of the MP optical density series tested. It would appear, therefore, that the MP optical density for a subject, in any given month, is largely unaffected by the optical density readings of the preceding month(s). The evidence suggests, rather, that monthly MP optical density fluctuates, in an apparently random fashion, about the mean level for each subject. However, as the literature germane to the stability of MP (over time) is limited, and as our data relates to just four subjects, firm conclusions should perhaps be deferred.

We report good interocular symmetry, with a mean difference of 0.043 for the group, which is again comparable with previously published data.[9,21,41–43] Moreover, changes in MP optical density over time exhibited interocular symmetry in three of the four subjects. For subject 1, the variation observed between both eyes was asymmetrical over the study period; however, this asymmetry can be attributed to just 4 of the 24 months' data. These results suggest that variations in MP optical density primarily reflect changes in non-ocular determinants of macular accumulation and stabilization of L and Z, rather than mechanisms within the individual eye.

Mean serum concentrations of L and Z were 0.086 (0.025) μg/ml and 0.032 (0.008) μg/ml, respectively, and are consistent with those obtained by previous investigators for populations of similar age groups.[5,33,43–45] As diurnal variation in plasma concentrations of total cholesterol and high-density lipoprotein cholesterol,[46] primary carriers of L and Z,[47] have been reported, it was important to investigate the diurnal variation, if any, of serum concentrations of L

and Z in our subjects. We found no statistically significant diurnal variation (8:30 a.m. to 6.00 p.m.) for serum concentrations of either L or Z, in any subject. In contrast, Cantilena et al.[48] reported significantly lower serum concentrations of L and Z at 5:00 p.m. when compared with measurements taken at other times in a given day but conceded that they could not satisfactorily explain their findings. However, as L and Z are fat-soluble carotenoids and are therefore likely to have a slow elimination half-life, the negligible diurnal variation in serum concentrations of L and Z that we observed is unsurprising. In brief, therefore, it appears that quantification of serum L and/or Z is not compromised if serum samples are taken at different time points in a given day.

Examining the series of serum L (and Z) of all four subjects, and using the diurnal ranges from Table 1 as indicators of measurement error, we can conclude that there is greater month-to-month variation in serum concentrations of L and Z than can be attributed to measurement error. These fluctuations may relate to perennial variability in dietary intake of carotenoid-containing foods,[49] and the carotenoid composition of those foods,[25,26] and/or changes in bioavailability and clearance of L and/or Z.[50–52]

For serum concentrations of L (and Z), subjects 1 to 4, all exhibited some variation in their monthly measurements, but the percentage variation was in no way comparable to that for MP optical density. For example, for subject 1, the maximum serum L concentration was just 4% above the mean level for this subject, and the minimum was only 2% below the mean level. As was the case with MP optical density, there were no outliers (i.e., at no point over the 24-month period did either serum L or Z exceed the range mean $\pm 3 *$ standard deviation for any subject). It appears, therefore, that in the absence of dietary modification and/or supplementation, there is relative monthly stability of serum L (and Z) concentrations.

It is important to note that (for typographical reasons) the MP optical density graphs in Figures 1–4 are on a different scale to that of serum concentration of L and Z. As a consequence, it appears that serum L (and Z) variation exceeds MP variation, whereas the opposite is in fact the case when variation is expressed as a percentage of the respective mean levels.

For subject 1, the monthly series of serum L concentrations demonstrated a small, but statistically significant, decline ($p = 0.049$) over the study period. This decline, obtained by regression analysis of serum L concentrations versus month (1, 2, 3 . . . 24), may be attributable to serial autocorrelation, as that subject's 2-month lag correlation was significant. In the presence of such autocorrelation, it is difficult to assess whether the underlying trend has a positive, zero, or negative slope. However, the mean serum L concentration for this subject declined from 0.107 for the first 12 months of the study, to 0.079 for the second 12 months. All other series, whether for serum concentrations of L or Z, were stationary, in terms of the mean and variance, over the study period. In contrast with the series of MP optical density measurements, there was evidence of autocorrelation (at lags 1 and 2 months) in some of the serum L and Z series, but not uniformly across subjects or series. For example, serum L concentrations taken 2 months apart for subject 1 were significantly correlated, but not 1 month apart; and serum Z concentrations for this subject were not significantly correlated at lags of either 1 or 2 months.

To date, there are three published studies that have investigated serum concentrations of L and/or Z with respect to the seasons. Of these, one study reported serum L concentrations to be higher in summer than in either autumn or winter in men, and higher in spring than in summer in women ($p < 0.05$).[15] These findings were not reproduced in our study, with the exception of subject 3 (female), whose serum L concentrations were higher in spring than in summer. Cooney et al.[53] found serum L concentrations to be higher in winter than in summer, and a further study failed to identify any relationship between serum concentrations of L (or Z) and the seasons.[48] Our data does, however, demonstrate a significant season effect on serum concentrations of L and Z but that this effect differs between subjects. In the context of our findings and these preceding and conflicting reports, it is difficult to detect an obvious pattern relating serum concentrations of L (or Z) with the seasons. In recent years, the availability of wide variety of foodstuffs throughout the year has become increasingly common, possibly attenuating any seasonal variations in serum concentrations of L and/or Z that may have existed in the past.

In the current study, we found that monthly fluctuations in serum concentrations of L were consistently accompanied by parallel changes in serum concentrations of Z, and that these relationships were statistically significant for three of the four subjects and approached statistical significance for the fourth ($p = 0.075$). Although,

several studies have reported the qualitative and quantitative content of carotenoids in fruits and vegetables, few have reported on the separate content of L and Z.[54–56] Therefore, we cannot conclude with certainty that the parallel changes we observed were solely due to diet. However, in the absence of dietary modification or supplementation, it is likely that the ratio of dietary L to dietary Z remained stable throughout the study period, thus explaining our observation.

Alternatively, other possible explanations for the parallel nature of serial serum concentrations of L and Z could relate to variations in the fat content of an individual's diet (and thus the ease at which the carotenoids are absorbed in the gastrointestinal tract),[57,58] changes in body fat (which is a known reservoir for L and Z),[9,20] and fluctuations in oxidant load (thus depleting both of these carotenoids in times of high oxidant load).[59]

Interestingly, the parallel changes we observed in serum concentrations of L and Z are not consistent with possible interconversion of these carotenoids occurring within the serum.[60] Of note, there is only evidence for these metabolic transformations occurring in the retina, and not in serum.[60,61] Further, a recent study in which rhesus monkeys, reared on carotenoid-free diets, were fed L and Z reported an absence of serum Z in the L-fed group, and only trace amounts of 3′didehydrolutein (a variant of L) in the Z-fed group, suggesting that such interconversion between L and Z in the serum is negligible.[62]

We observed no obvious relationship, synchronous or lagged, between monthly MP optical density and monthly serum L (and/or monthly serum Z) concentrations, for any subject. This finding is not surprising as serum concentrations of L (and Z) reflect recent nutritional intake only,[22] whereas MP has a biological turnover that is slow, and which may differ between individuals.[12] Also, our sample size is a limiting factor ($n = 4$), and all correlations were carried out within subjects in whom no statistically significant peaks or troughs of MP optical density, or serum concentrations of L or Z, were demonstrable during the 24-month study period. In other words, and although we did observe fluctuations in serum concentrations of L and Z, these changes were minute when compared with those of supplementation studies, which are typically accompanied by parallel change in MP optical density in a majority of subjects.[4,12,63,64] Of eight cross-sectional studies investigating the relationship between serum concentrations of L (and Z) and MP optical density at a single point in time, six have reported positive and significant correlations ($p < 0.05$; $r = 0.21$ to 0.82).[9,14,16,34,43,65–67]

In conclusion, we report month-to-month fluctuations in MP optical density, and serum concentrations of L and Z, which cannot be attributed to measurement error but that do not reach statistical significance. Diurnal variation of these parameters was negligible. Seasonal effects were present for serum L and for serum Z, but these effects differed between subjects. Fluctuations in serum concentrations of L and Z were synchronous and parallel but were unrelated to fluctuations in MP optical density. In the absence of dietary modification or supplementation, MP optical density, and serum concentrations of its constituent carotenoids, are relatively stable.

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