Measurement of Macular Pigment: Raman Spectroscopy versus Heterochromatic Flicker Photometry

Kumari Neelam,[1,2] *Nicholas O'Gorman,*[1] *John Nolan,*[1] *Orla O'Donovan,*[1] *Hwee Bee Wong,*[3] *Kah Guan Au Eong,*[4] *and Stephen Beatty*[1,2]

Purpose. There are several techniques for measuring macular pigment (MP) in vivo, of which Raman spectroscopy (RS) is a recently developed objective method. This study reports the reproducibility, test–retest variability, and validity of RS MP readings, by comparing them with heterochromatic flicker photometry (HFP).

Methods. MP was measured with HFP and RS in 120 healthy subjects, and the latter technique was also used on two separate occasions in a sample of 20 subjects to investigate the intersessional variability of readings. Intrasessional reproducibility of RS MP measurements was also calculated. In addition, serum concentrations of lutein (L) and zeaxanthin (Z) were measured and correlated with both RS and HFP MP readings.

Results. Mean (±SD) MP in the right eye was 0.279 ± 0.145 and 0.319 ± 0.155 with RS and HFP, respectively. The differences between corresponding MP readings taken on RS and HFP lay within the Bland-Altman 95% limits of agreement for the two instruments in 93.6% and 94.4% of cases in the right and left eyes, respectively. Intrasessional reproducibility of RS readings, expressed as the coefficient of variation, was 8.42% ± 7.12%. Ninety-five percent of MP readings taken with RS on two separate occasions lay within the 95% limits of agreement for the two sessions. A positive, but insignificant, relationship was observed between RS and HFP MP readings and serum concentrations of L and Z (RS, $P = 0.356$; HFP, $P = 0.540$).

Conclusions. RS, an objective method of measuring MP levels in vivo, exhibits acceptable reproducibility and test–retest variability. The results demonstrated good correlation between RS and HFP measurements of MP, thus authenticating RS against a validated psychophysical technique of measuring MP. However, investigators should use only one of these instruments for the duration of any given study because of differences in the scientific rationale, and the factors that influence RS and HFP measurements of MP. (*Invest Ophthalmol Vis Sci.* 2005;46: 1023–1032) DOI:10.1167/iovs.04-1032

Macular pigment (MP), composed of two dietary xanthophylls, lutein (L) and zeaxanthin (Z), forms a characteristic yellow spot in the center of the primate macula.[1] The spatial distribution of MP varies across the retina, with a peak density in the central 1° to 2°, and declines to negligible levels by 5° to 10° eccentricity.[2] Within the layer structure of the retina, MP is maximally concentrated in the Henle fiber layer of the fovea.[3]

It has been hypothesized that MP may play a role in the prevention of age-related macular degeneration, the leading cause of blindness in the Western world, by acting as an optical filter to phototoxic blue light and/or as a consequence of its antioxidant properties.[4,5] In addition, a protective role of MP for other ocular diseases etiologically linked to oxidative stress, such as retinitis pigmentosa, has also been suggested.[6,7]

Therefore, the need for an objective, accurate, and rapid technique for measuring MP in vivo, which is also both sensitive and specific, is essential to researchers if the putative protective role of the macular xanthophylls is to be investigated.

Currently, the most widely used technique for measurement of MP is a psychophysical method known as heterochromatic flicker photometry (HFP).[2,8–10] This technique has been validated against in vitro measurements, and corrects for prereceptorial optical properties because it is derived from a ratio within the individual eye.[4] The requirements of this test include the need for an alert and cooperative subject with good visual acuity. The major limitations of HFP rest on the fact that it is a subjective technique that is time consuming and is associated with high variability in subjects with low MP optical density.[11]

Several objective techniques for measuring MP exist, each with its own merits and limitations. Raman spectroscopy (RS), which is based on the principle of the Raman shift and utilizes an argon laser to quantify MP, represents one such technique and is relatively new.[12,13] MP can be measured rapidly by RS in a clinical setting, even in subjects with macular disease, if central fixation is intact.[14]

In this article, we report the reproducibility and test–retest variability of RS measures of MP and compare these readings with those obtained with HFP in 120 subjects.

Methods

In this study, we recruited 125 healthy volunteers aged between 20 and 60 years, by a self-selected sample from the general population. Of the 125 subjects, 5 were unable to perform HFP, and therefore the data for 120 subjects were used for the purpose of statistical analysis. Further, of the 120 subjects, only one eye of six subjects was included in the study because the fellow eye of these subjects was ineligible as a result of ocular disease (e.g., amblyopia). In other words, there were 118 right eyes, and 116 left eyes of 120 subjects in the study.

In all participants, the best corrected visual acuity was 0.2 or better using the logarithm of the minimum angle of resolution (logMAR) visual acuity chart, and there was no evidence of visually significant cataract and/or macular disease on anterior and posterior segment photography, respectively. Informed consent was obtained from each volunteer, and the study protocol adhered to the tenets of the Declaration of Helsinki.

Comparison of RS and HFP

The MP was measured in each subject with HFP and RS on two separate occasions, with the interval between sessions not exceeding

From the [1]Waterford Institute of Technology, Waterford, Republic of Ireland; the [2]Waterford Regional Hospital, Waterford, Republic of Ireland; the [3]Clinical Trials and Epidemiology Research Unit, Singapore; and the [4]Alexandra Hospital, National Healthcare Group, Singapore.

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Corresponding author: Kumari Neelam, Department of Ophthalmology, Waterford Regional Hospital, Dunmore Road, Waterford, Republic of Ireland; neelamk@sehb.ie.

2 weeks. In addition, 6 to 8 mL of blood was collected from the subjects for quantification of L and Z in the serum.

Raman Spectroscopy

For assessment of reproducibility and test–retest variability of RS measurements, we recorded MP levels with the Raman spectrometer in 20 healthy volunteers during two sessions separated in time by at least 24 hours (but no more than 2 weeks).

Intrasessional Variability. The reproducibility (intrasessional variability) of the RS measurements was expressed as the coefficient of variation using the highest three readings recorded during a single session.

Intersessional Variability. Test–retest variability (intersessional variability) of RS measurements was assessed by comparing MP readings (the highest three, as well as all five) recorded during two separate sessions.

Measurement Protocol for HFP

MP was measured psychophysically with the Maculometer (developed by John Mellerio, School of Biosciences, University of Westminster, London, UK), which utilizes the principle of HFP.

Principle. HFP is based on the principle of matching the luminance of two flickering light sources, one blue and one green, at the fovea and then at the parafovea. If the green light remains at constant luminance while the luminance of the blue light is varied, a point of minimum flicker is achieved when the luminance of the two light sources are matched. The logarithm ratio of the luminances of blue light required to achieve this end point, for foveal and parafoveal readings, is a measure of the optical density of MP. This is because MP is optically undetectable at an eccentricity of 6.5°, and has peak absorption at 460 nm, corresponding to blue light.

Apparatus. The Maculometer is a small, portable instrument that uses light emitting diodes (LEDs) as light sources. LEDs provide good light sources for portable instruments because they are small, inexpensive, easily driven from simple power supplies, and emit near monochromatic light.

The test stimulus consists of a 1° circular dot (foveal), flickering between a 460-nm measuring field (blue light; peak MP absorbance) and a 560 nm reference field (green light; minimal MP absorbance). This is surrounded by two arcs representing a parafoveal annulus (diameter, 10°; width, 1°) concentric with the fovea, also consisting of a flickering stimulus composed of the same wavelengths as the foveal stimulus. The measuring and the reference fields are superimposed and presented out of phase at an alternating rate of 11 to 12 Hz and 6 to 7 Hz at the fovea and parafovea, respectively.

The luminance of green light is fixed but the subject, using a dial, can alter the luminance of the blue light. Flicker is obvious when the perceived luminance of the blue and green lights differs, but is minimal when these luminances are matched.

Procedure. Subjects were given brief instructions on the method, and a practice trial before actual readings were recorded. Subjects used their corrective lenses, and near visual acuity was 20/25 or better in all eyes tested.

For foveal matches, the subject was asked to look at a flickering blue/green light in the central field with parafoveal arcs extinguished and to reduce the flicker to a minimum by adjusting the blue light with the help of the dial. The subject's perception of the end point was then recorded. For parafoveal matches, the foveal field was changed from its flickering status to a dim red light to provide a fixation target while the parafoveal arcs were flickering. The entire procedure was then repeated, with the test field imaged at the parafovea.

Perfectionist adjustment of the control was strongly discouraged, as the point of no flicker cannot be achieved. After recording of each reading, the investigator set the luminance control to some new arbitrary position so that the subject could not learn how far to adjust the dial to obtain a match. Between four and eight readings were recorded at the fovea and the parafovea for each eye and then used to calculate MP optical density.

Measurement Protocol for Resonance RS

We also used resonance Raman scattering spectroscopy, a novel, noninvasive, and objective method employing laser technology, to measure MP densities in all subjects.

Principle. RS is based on the Raman effect/shift, which is the inelastic scattering of photons by the molecules under investigation. In other words, the wavelength of a small fraction of the radiation scattered by certain molecules differs from that of the incident beam, and the shift in wavelength depends on the chemical structure of the molecules responsible for the scattering. This phenomenon can be used to quantify MP because macular carotenoids, when excited with monochromatic laser beam, exhibit characteristic wavelength shifts of the back-scattered light that are generated by vibrational modes in their chemical bond.

Raman scattering is a very weak optical effect and does not require resonant excitation. However, when a molecule is illuminated with monochromatic light overlapping its absorption band, then the Raman scattered light exhibits a substantial resonance enhancement. In the case of a carotenoid molecule, a 488-nm argon laser light provides an extraordinarily high resonance enhancement of Raman signals of five orders of magnitude.

The Raman peaks are highly specific for a carotenoid molecule because their spectral locations correspond exactly to the vibrational energies of the Raman active bonds within the molecules and have specific relative intensities. The chance of the same spectral peaks originating from another molecule within the retinal pigment epithelium or blood is virtually negligible, because each molecule would have different bonds (vibrational energies), resulting in Raman peaks at different frequencies, compared with the carotenoid Raman peaks.

Apparatus. The portable instrument consists of a laser source, light delivery and collection module, and a spectrograph coupled with a charge-coupled device (CCD; silicone chip) camera and associated electronics.

The excitation laser beam originates from the air-cooled argon laser of 488 nm and an eyepiece–lens–filter combination system projects it onto the macula via a fiberoptic light delivery bundle. The Raman scattered light is collected (180° backscatter geometry) in a fiberoptic collection bundle (aperture size, 7 mm) with the same system used to deliver the excitation light, but with a separate light-collection path. The dispersed spectrum is analyzed by Raman spectrograph and imaged on the CCD camera. The instrument is interfaced with a computer for data acquisition and processing.

There are various ingrained provisions to aid proper optical alignment of the instrument with the human eye. A blue spot, originating from the end face of the excitation light delivery fiber, is visible even in a closed state, because of the unique design of the optical shutter, which allows a very small portion of the blue argon laser light to be transmitted all the time. In addition, a red polka-dot pattern, originating from the facets of the light collection fiber bundle illuminated with LED, is visible as an alignment aid for the subject.

For a typical measurement, a 1-mW argon laser beam is projected as a 1-mm spot onto the subject's macula for 0.25 seconds. The peak height at the carotenoid carbon–carbon double bond stretch frequency of 1525 cm^{-1} is used as a measure of carotenoid concentration after subtraction of the background fluorescence by Windows-based computer software (Eye-C-Spec; Spectrotek, LC, Salt Lake City, UT), and the Raman signal intensity is expressed as photon counts. The instrument provides an almost instantaneous display of final Raman spectrum, as postexposure processing takes only 0.25 seconds.

Procedure. All measurements were taken in a dark room after maximum pupillary dilation with 1% tropicamide. Subjects were asked to overlap the blue disc and red polka-dot pattern to ensure proper alignment of the eye with respect to the instrument. Subjects with significant refractive error wore their usual spectacle or contact lens corrections.

After the subject confirmed optimal alignment, the subject was asked to fixate in the center of the test field to ensure foveal fixation, and then the operator triggered signal capture by means of a button.

The synchronized system of electronics momentarily switched off the red LED–aiming beam, opened the shutter to allow laser light to project onto the retina, and triggered the data-acquisition system.

Five measurements were recorded in each eye at intervals of 2 to 3 minutes to allow the flash afterimage to fade. Because subjects occasionally blinked or misaligned, the three highest of these five measurements were used for data analysis.

High-Performance Liquid Chromatography for Serum Analysis of L and Z

High-performance liquid chromatography (HPLC) on a reversed phase column is a powerful technique for separation, identification, and quantification of various forms of carotenoids in human serum and other tissues.

Instrumentation. We used a system (HP 1090 LC; Hewlett-Packard, Palo Alto, CA) with photodiode array detection at 292, 325, and 450 nm under computer control (Chem Station software; Agilent, Palo Alto, CA). A 5-μm analytical–preparative 4.6 $\times$ 250-mm specialty reversed phase column (201 TP; Vydac, Hesperia, CA) was used with an in-line guard column. The mobile phase, consisting of 97% methanol and 3% tetrahydrofuran, was degassed with an in-line degasser. The flow rate was 1 mL/min. Hoffmann-La Roche (Nutley, NJ) provided the standards for HPLC analysis.

Procedure. Blood samples (6–8 mL) were collected in two 5-mL tubes (Vacutainer; BD Biosciences, Franklin Lakes, NJ) containing 4.5 U of sodium or lithium heparin per milliliter of whole blood, and immediately centrifuged at 5000 rpm for 10 minutes. The separated serum layer was then aliquoted into three light-sensitive microcentrifuge tubes and stored at −70°C until the time of analysis.

Extraction. A 0.4-mL aliquot of serum was pipetted into light-sensitive microcentrifuge tubes (2 mL total capacity). Ethanol (0.3 mL) containing 0.25 g/L butylated hydroxytoluene and internal standard (tocopherol acetate) was added to each tube. Heptane (0.5 mL) was then added, and samples were vortexed vigorously for 1 minute followed by centrifugation at 2000 rpm for 5 minutes (MSC Micro Centaur; Davison & Hardy Ltd., Belfast, UK). The resultant heptane layer was retained, transferred to a second labeled light-sensitive microcentrifuge tube, followed by a second heptane extraction. The combined heptane layers were immediately evaporated to dryness under a stream of pure nitrogen, using a sample concentrator (Techne, Cambridge, UK). These dried samples were reconstituted in methanol (200 μL), and 150 μL was injected for HPLC analysis. The total run time was 15 minutes.

L and Z standard curves were used for quantification of serum concentrations of these carotenoids for each subject. This assay has been validated against the National Institute of Standard and Technology (NIST) Standard Reference Material 968c for Carotenoids in Human Serum.

Data Analysis

The data were analyzed on computer (SPSS statistical software; SPSS, Chicago, IL). Univariate analysis was conducted with the Mann-Whitney test and Wilcoxon signed-rank test, because the normality assumption needed for parametric tests (two-sample *t*-test and paired *t*-test) was not satisfied.

Correlations were evaluated by linear regression analysis, and multiple linear regression models were used to assess cross-sectional relationships. $P < 0.05$ was deemed significant.

For comparison with HFP, RS MP readings were standardized by dividing the mean of the three readings by 4000, assuming ranges of −500 to 3500 and 0 to 1 for Raman and HFP, respectively. In other words, for comparison purposes, RS MP readings were converted arbitrarily to optical density measurements. Differences between corresponding HFP and Raman readings were calculated, the mean and standard deviation of these differences were used to calculate the 95% limits of agreement between the two instruments (mean ± 1.96 SD), and Bland-Altman plots were generated.

Intrasessional reproducibility and intersessional variability of RS readings were expressed as the coefficient of variation and shown in Bland-Altman plots, respectively.

Results

In this study, we enrolled 120 healthy volunteers to compare two techniques for measuring MP, HFP, and RS. The mean age of our study population (±SD) was 40.89 ± 12.08 years (range, 22–60). Of the 120 subjects, 73 (60.8%) were women and 47 (39.2%) were men.

Raman Spectroscopy

The mean level of MP (±SD) using RS was 1118 ± 580 and 1128 ± 626 in the right and left eyes, respectively (Fig. 1). There was a good degree of interocular agreement in MP levels (mean difference 2.37 [±324.94]), with a maximum right–left eye difference of 804 (Wilcoxon signed rank test; $P = 0.669$).

Multiple linear regression was used to analyze the relationship between MP and other variables such as age, gender, and pupil size, and revealed a significant relationship between RS MP readings and age ($P = 0.004$) and pupil size ($P = 0.006$).

There was no statistically significant difference in mean MP (±SD) levels between men and women (men: $n = 45$; right eye, 1190 ± 575; women: $n = 73$; right eye, 1073 ± 583; Mann-Whitney test, $P = 0.26$). Subjects aged <55 years had significantly greater levels of mean MP than did subjects older than 55 years (subjects <55 years: $n = 97$; right eye: 1218 ± 567; subjects aged ≥55 years: $n = 21$; right eye, 655 ± 394; Mann-Whitney test; $P < 0.001$). In other words, there was a 448-unit increase in RS MP readings in subjects aged <55 years compared with those aged ≥55 years.

Subjects with pupil diameter of ≥7 mm had significantly higher levels of mean MP (±SD) than those with pupil diameter <7 mm (pupil size, ≥ 7 mm: $n = 65$; right eye, 1247 ± 517; pupil size < 7 mm: $n = 22$; right eye, 792 ± 478; Mann-Whitney test, $P < 0.001$; Fig. 2). In other words, there was a 356-unit increase in RS MP readings in patients with a pupil size of ≥7 mm compared with those with a pupil size of <7 mm.

Using RS, MP was measured in one subject before and after pupil dilation of greater than 7 mm. We found an average increase of 516 units in the right eye and 400 units in the left eye with dilated pupil, when compared with the undilated pupil (right eye, $P = 0.003$; left eye, $P = 0.130$).

However, within subjects with pupil diameter greater than 7.0 mm (right eye, $n = 65$; left eye, $n = 64$), pupil size was unrelated to MP levels in either eye. Pupil size data were unavailable for 31 subjects.

Heterochromatic Flicker Photometry

The mean level of MP (±SD) using HFP was 0.319 ± 0.155 and 0.321 ± 0.156 in the right and left eyes, respectively. There was a good degree of interocular agreement in the MP level of mean difference (0.0054 [±0.1020]), with a maximum right-left eye difference of 0.222 (Wilcoxon signed rank test; $P = 0.897$).

There was no statistically significant difference in mean MP (±SD) levels between men and women (men: $n = 45$; right eye, 0.338 ± 0.152; women, $n = 73$; right eye, 0.307 ± 0.156; Mann-Whitney test, $P = 0.355$). Subjects aged <55 years had greater levels of MP than subjects aged ≥55 years (subjects <55 years, $n = 97$; right eye, 0.329 ± 0.148; subjects ≥55 years, $n = 21$; right eye, 0.269 ± 0.178), but this difference did not reach statistical significance (Mann-Whitney test; $P = 0.188$).

Comparison of RS and HFP

The mean levels of MP (±SD) recorded using RS were 0.279 ± 0.145 and 0.282 ± 0.156 in the right and left eyes, respectively.

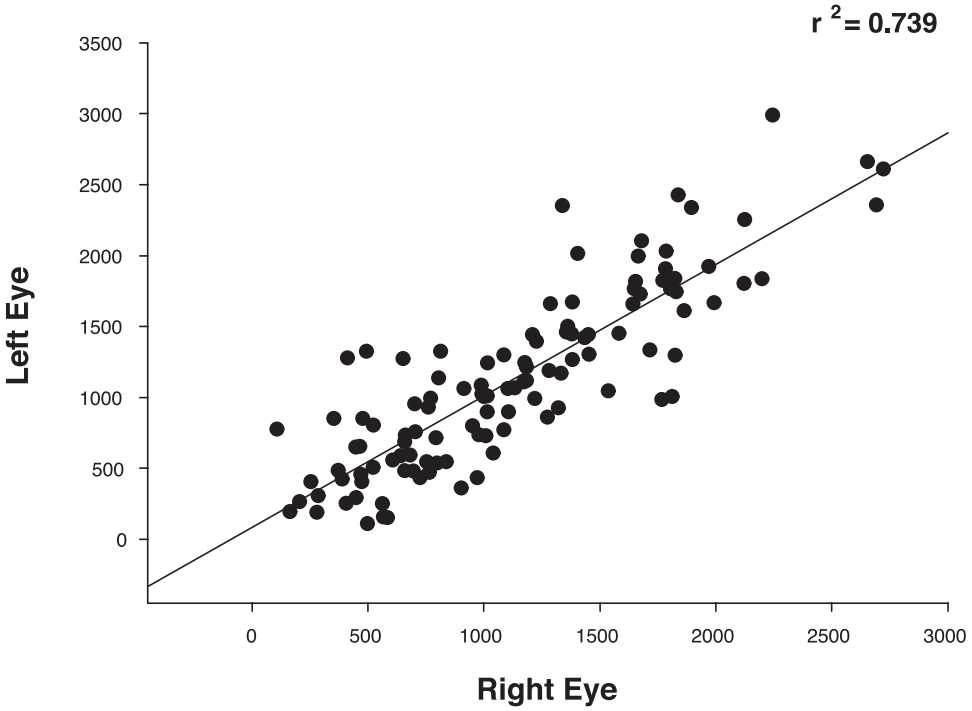

FIGURE 1. RS MP readings in the right and left eyes.

The corresponding values for HFP were 0.319 ± 0.155 and 0.321 ± 0.156 in the right and left eyes, respectively.

It was observed that the difference between corresponding MP readings taken by RS and HFP lay within the 95% limits of agreement for the two instruments in 93.6% and 94.4% of cases for the right and left eyes, respectively (Fig. 3). Of the eight outliers in the right eye, four were encroaching on the 95% limits of agreement. Of the seven outliers in the left eye, two were very close to the 95% limits of agreement.

There was a significant age-related decline in MP observed with RS and HFP (Fig. 4; linear regression. RS: $P = 0.0001$, $r = -0.433$; HFP: $P = 0.048$, $r = -0.181$). However, when we excluded study eyes in which pupil diameter was unknown

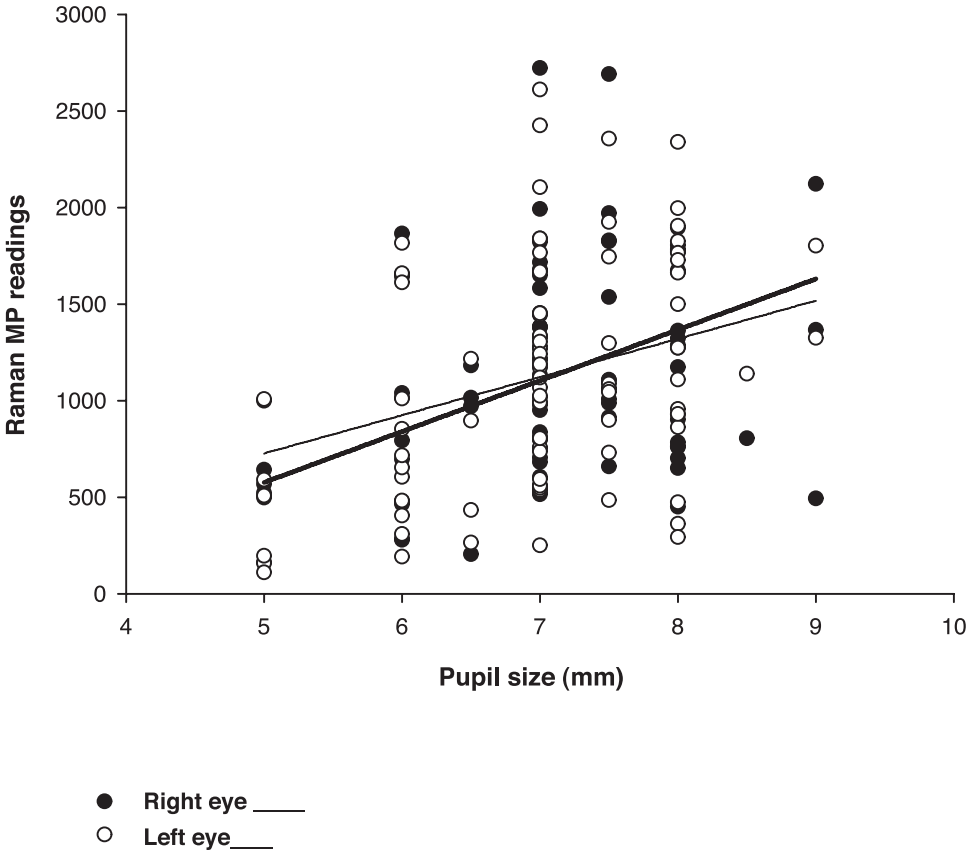

FIGURE 2. Correlation between RS MP readings and pupil size in the right (●, *light solid line*) and left (○, *heavy solid line*) eyes.

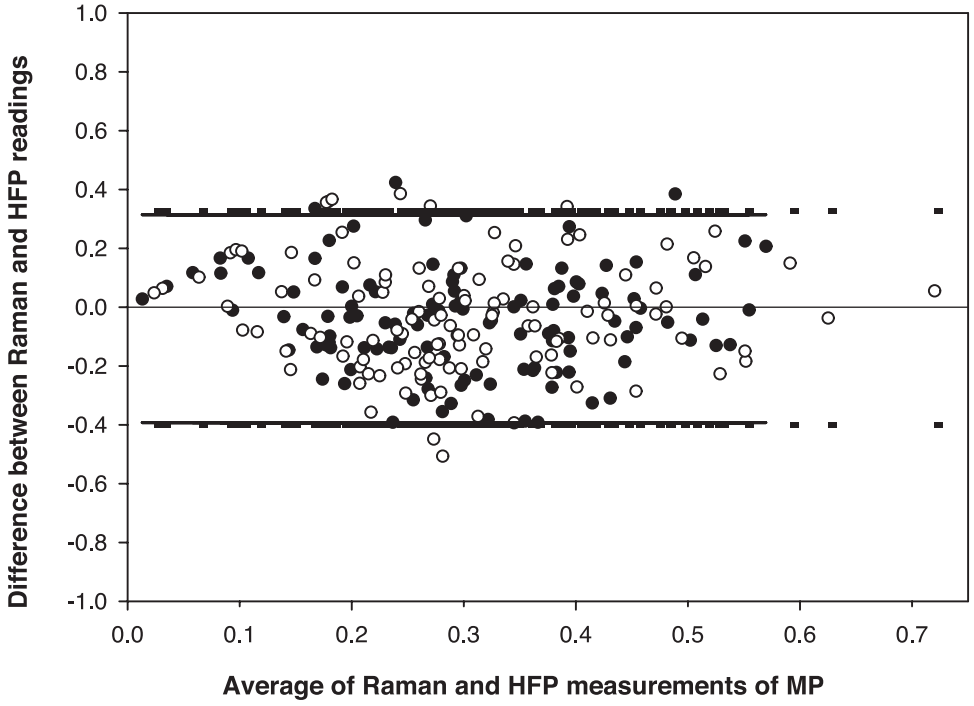

FIGURE 3. Bland-Altman plot showing 95% limits of agreement between RS and HFP measurement of MP in the right (●) and left (○) eyes.

(n = 31) or <7 mm (n = 22), the age-related decline was dramatically attenuated to nonsignificance for MP readings using RS (preexclusion $P = 0.0001$, $r = -0.433$; postexclusion $P = 0.313$, $r = -0.127$) and minimally attenuated for MP readings using HFP (preexclusion $P = 0.048$, $r = -0.181$; postexclusion $P = 0.063$, $r = -0.231$; Fig. 5).

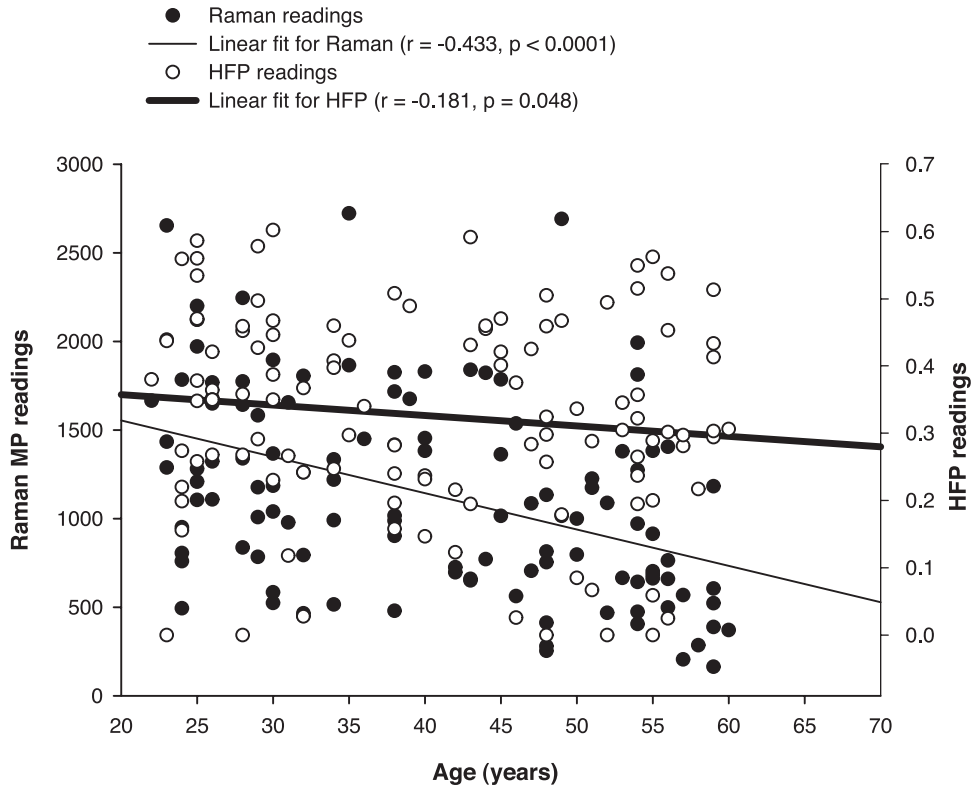

FIGURE 4. Age-related decline in MP levels, as shown by RS and HFP.

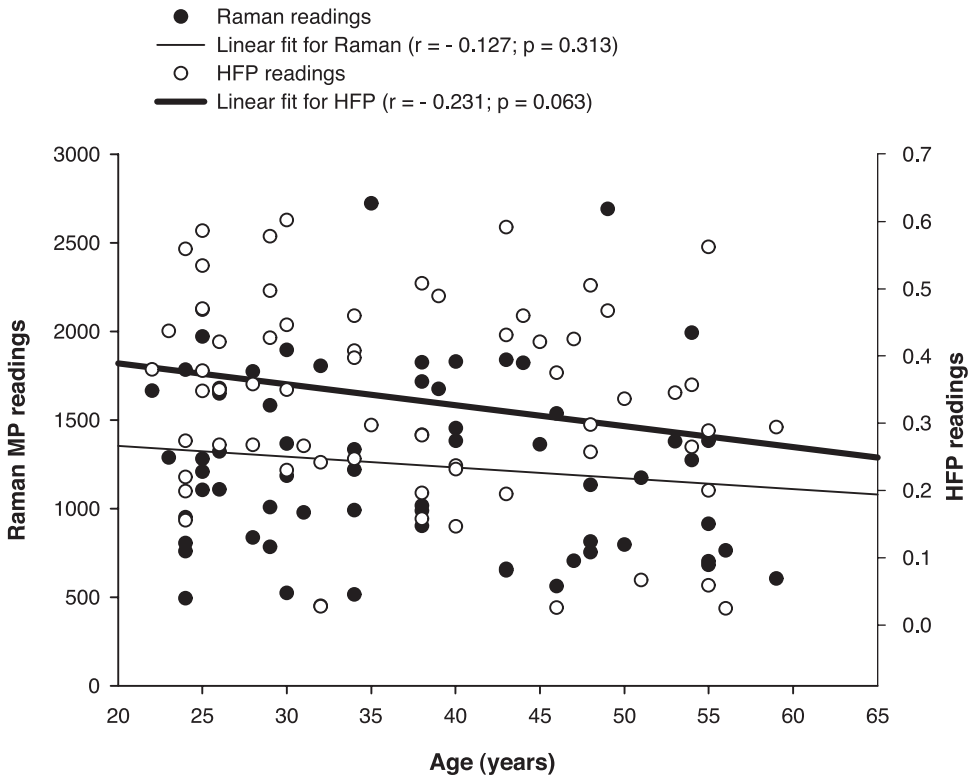

FIGURE 5. Age-related decline in MP levels, as shown by RS and HFP in subjects with pupil diameter ≥7 mm.

Intrasessional Reproducibility of RS Readings

The mean (± SD) coefficient of variation was 12.61% ± 9.46% and 8.42% ± 7.12% with a maximum difference of 42.25 and 30.10 on days 1 and 2 respectively, in 20 healthy subjects.

Intersessional Reproducibility of RS Readings

Ninety-five percent of MP readings taken with RS on two separate occasions lay within the 95% limits of agreement for the two sessions (Figs. 6, 7).

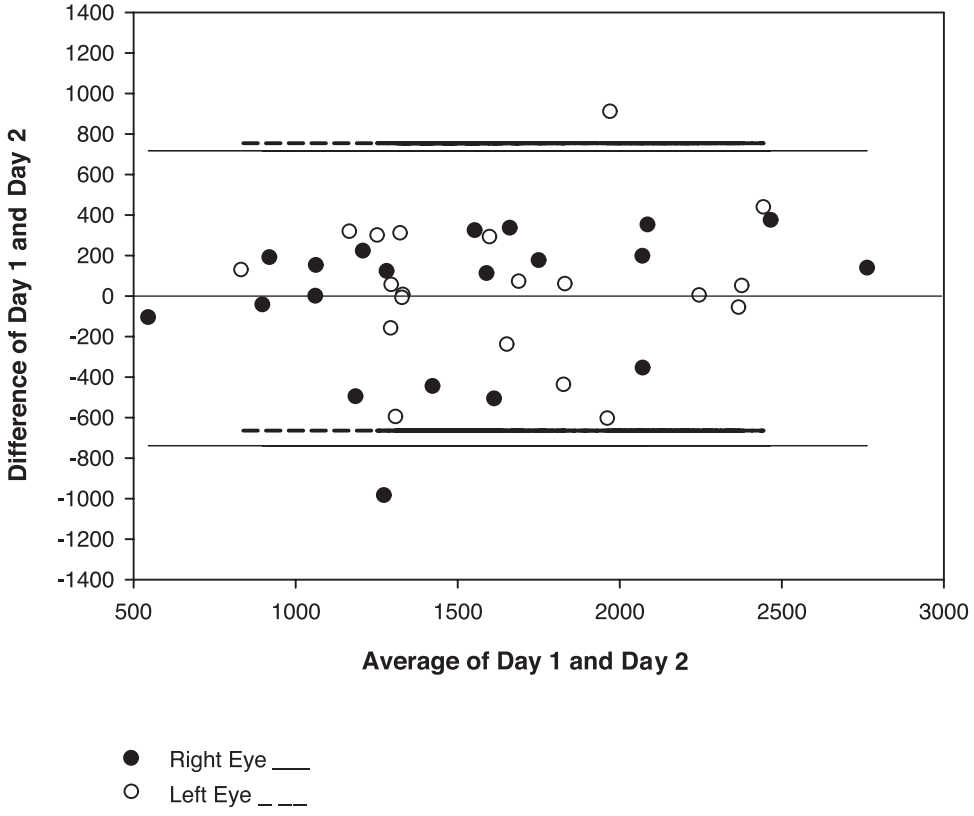

FIGURE 6. Bland-Altman plot showing test–retest variability of top three RS readings in the right (●, *solid line*) and left (○, *dashed line*) eyes of 20 subjects.

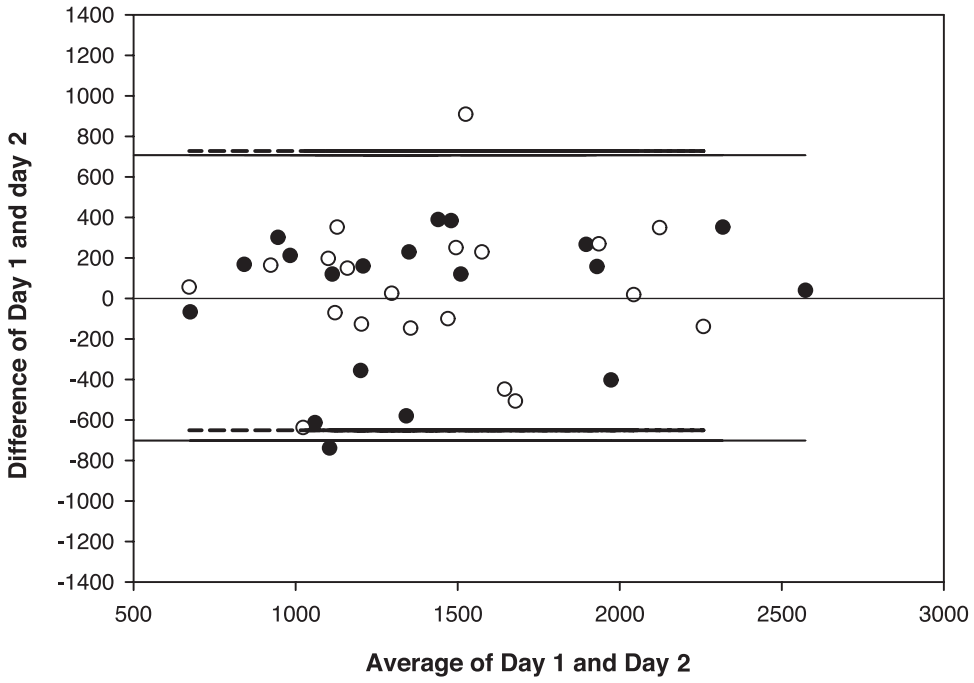

FIGURE 7. Bland-Altman plot showing test–retest variability of all five RS readings in the right (●, *solid line*) and left (○, *dashed line*) eyes of 20 subjects.

Correlation of Serum Levels of L and Z with RS and HFP MP Readings

The mean (±SD) serum levels of L (Fig. 8) and Z (Fig. 9) were 0.086 ± 0.043 μg/mL and 0.026 ± 0.014 μg/mL, respectively. The serum concentrations of L and Z correlated more strongly with RS MP levels than with HFP readings, but did not reach statistical significance with either instrument.

DISCUSSION

In this study, we measured MP in 120 healthy subjects with RS and HFP, for purposes of comparing these two techniques of evaluating levels of this pigment in vivo. In addition, we tested the reproducibility and test–retest variability of RS MP measurements in 20 healthy subjects. The data from this study demonstrate that MP levels measured with RS show a good degree of correlation with the corresponding HFP readings. Furthermore, RS measurements of MP are highly reproducible and are not subject to meaningful test–retest variability.

Over the past few years, there has been a growing need to measure MP to investigate its putative protective role for retinal disease, and several objective techniques have been explored with variable success.[15–17] Bernstein et al.[18] have recently developed a novel optical approach that is also objective for measuring MP in living primates based on Raman scattering, and this method is known as RS.[18]

Using RS, we measured MP in 120 subjects and recorded mean levels that were higher (1123 ± 603) than those recorded in 76 subjects of similar age profile from Salt Lake City, Utah (685 ± 447).[19] The reason for this discrepancy remains unclear but may reflect differences between the two populations studied (American versus European) in terms of dietary and ethnic factors. There was excellent interocular symmetry of MP levels obtained by RS and HFP in our subjects, consistent with the findings of Bernstein et al.[20] and with data for MP assessed with HFP.[14,19,21]

There was no significant influence of gender on MP levels measured by RS, consistent with previous studies using this technique.[14,19,20] Similarly, there was no significant difference in MP optical density between men and women when measured with HFP. However, results from some, but not all, studies in which HFP was used to measure MP showed a relative lack of the pigment in women when compared with men.[22]

The marked age-related decline in MP levels reported by investigators using RS has been a topic of debate in the literature.[23,24] Our results have shown that subjects aged ≥55 years have less MP than subjects <55 years, irrespective of the method used for measuring MP. This finding is consistent with the work of previous investigators employing RS for MP measurements,[14,19,20] but less consistent with the literature reporting other techniques for evaluation of MP.

HPLC analysis of MP in 87 donor eyes by Bone et al.[25] did not demonstrate an inverse relationship between MP optical density and age. Of the seven psychophysical studies, four demonstrated an age-related decline in MP optical density, and this relationship reached statistical significance in two of the studies.[10,26–31] Of the eight studies in which the fundus reflectance technique was used for measurement of MP, two showed an age-related decline in MP.[15,16,32–37] However, all three studies involving the autofluorescence technique for measurement of MP failed to show an age-related decline.[16,35,38]

It is evident, therefore, that there is no shortage of studies in the published literature that have reported on the relationship between age and MP levels. However, it is difficult to draw a firm conclusion regarding age's effect on MP because of inconsistencies in the results. Clearly, and in the context of substantial individual variability in MP optical density, a large

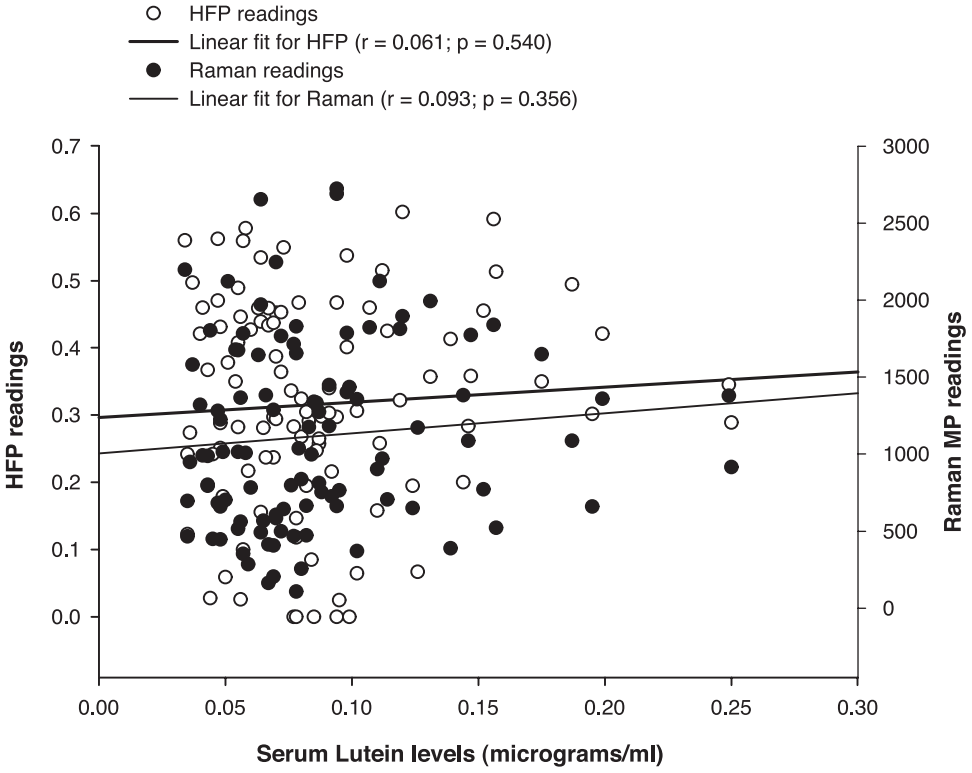

FIGURE 8. Correlation of serum lutein levels with RS MP and HFP readings.

number of subjects is needed for any such investigation. Ultimately, a longitudinal study of MP measurements is necessary to address the question of whether MP optical density is affected by age.

In our study, the age-related decline in MP observed using RS ($P = 0.0001$; $r = -0.433$) was much greater than that observed using HFP ($P = 0.048$; $r = -0.181$). This discrepancy may be attributable to the recently reported increase in the lateral extent of MP with age (Delori FC, et al. *IOVS* 2004;45: ARVO E-Abstract 1288), which may artifactually demonstrate an age-related decline in the pigment as measured by HFP, but would have no such effect on RS, which measures the pigment

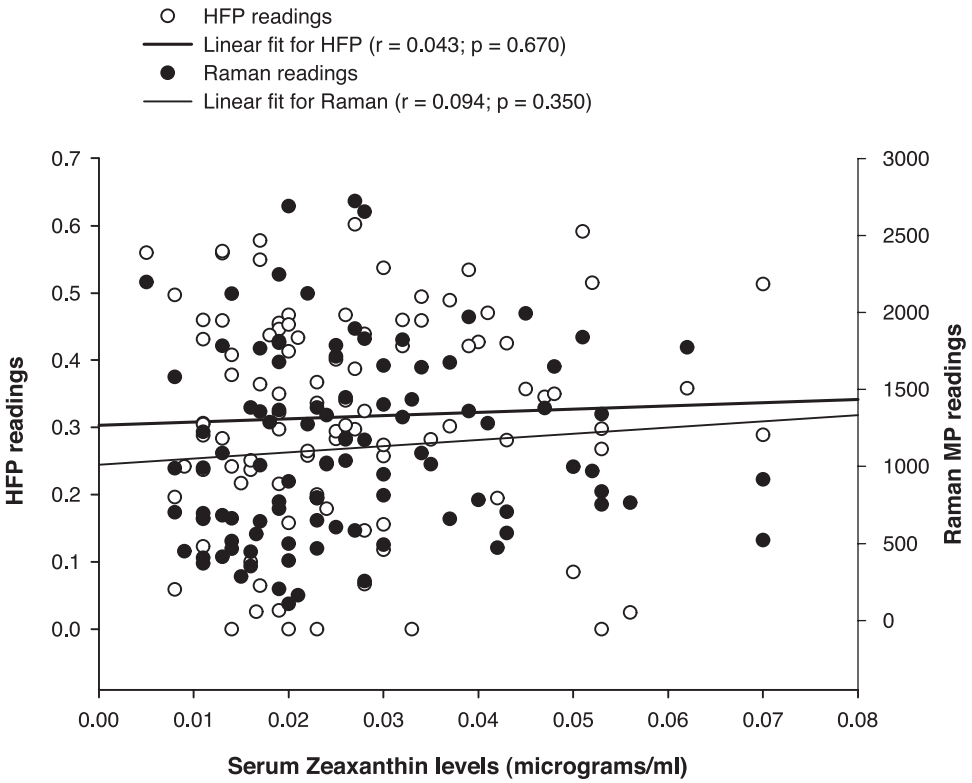

FIGURE 9. Correlation of serum zeaxanthin levels with RS MP and HFP readings.

at the central 1 mm of the fovea only. Alternatively, the age-related decline in MP may be exaggerated when measured with RS, because of absorption and/or diffusion (aberrations plus intraocular scatter) related to visually inconsequential cataracts or other incipient age-related factors.[24]

The spatial distribution of MP in the primate fovea varies between individuals, and may affect its measurement. Although there is a positive correlation between peak density of MP and its spatial half-width and asymptote, this relationship decreases as the eccentricity of the reference location is reduced.[2] In addition, with age, there is an extension of the lateral extent of MP, perhaps without a significant change in its peak density.[32] Therefore, it is likely that the central peak concentration represents only an approximate reflection of the total amount of MP present, and our findings must be interpreted with full appreciation of this limitation.

An age-related decline in MP, if any, may be attributable to excessive depletion or inadequate uptake of the macular carotenoids with increasing age. A depletion of MP with age would be consistent with excessive utilization of L and/or Z, or coantioxidants such as ascorbate and α-tocopherol, in response to the age-related increase in oxidant load.[39,40] The inadequate uptake of macular carotenoids in association with increasing age could be attributable to age-related changes in dietary intake, absorption, transport in serum, and/or capture by retinal tissue.[10]

Our data fully support the previous finding that pupil size significantly affects the measurement of MP levels with RS.[41] The measured signals were noticeably lower in subjects with pupil diameter <7 mm when compared with the levels for pupil diameter ≥ 7 mm. However, Raman measurements of MP were independent of pupil size when pupil diameter was >7 mm, above which the aperture of the light-collection fiber (and not the pupil) is the limiting factor.

Indeed, when we excluded study eyes for which pupil diameter was unknown or <7 mm, the age-related decline was dramatically attenuated to nonsignificance for MP readings using RS, and minimally attenuated for MP readings using HFP. This attenuation of the age-related decline of MP as measured by RS is consistent with the view that age-related miosis may contribute, at least in part, to the decline in levels of pigment with increasing age observed by investigators using RS.

Of note, MP levels measured with RS are not directly comparable with the readings obtained with HFP, because the Raman technique measures the total carotenoids in the entire region illuminated by a 1 mm spot, whereas HFP measures optical density of MP at the edge of the illuminated spot.[2] However, the results from the present study indicate that there is a good degree of correlation between MP readings recorded with RS and with HFP, and suggest that the amount of pigment in the central 1 mm area of the fovea may reflect the peak MP optical density.

In our study, agreement between the two methods for measuring MP—RS and HFP—as shown in Bland Altman plots, was acceptable (right eye, 93.6%; left eye, 94.4%), but failed to reach the statistically significant level of 95%. It is worth mentioning, however, that the two techniques would be statistically interchangeable if the outliers (all of which were encroaching on the 95% limits of agreement) were considered to lie within the acceptable range. Relating our findings with those in previous reports of comparisons between MP readings taken on these two instruments would not be meaningful, because Bland-Altman plots have not been generated for this purpose by previous investigators (Wintch SW, et al. *IOVS* 2003;44:ARVO E-Abstract 2551).

This is the first study to investigate the relationship between serum concentrations of L and Z with MP readings obtained by RS. A positive, albeit insignificant, relationship between MP and serum concentrations of its constituent carotenoids was found for both instruments. Of eight previous studies investigating this relationship, seven have found a positive and significant correlation, including a recent article published from our laboratory.[30,42] In the present study, we believe that the correlation did not reach statistical significance simply because of the narrow range of the serum concentrations of the macular carotenoids in our sample. Certainly, this view is consistent with the lack of a demonstrably significant relationship with both instruments. The data demonstrate, however, that the correlation was stronger for RS MP readings than for HFP readings.

Whether two instruments designed to measure the same variable are interchangeable depends on the measurement in question, and the use to which it is put. In other words, it is a clinical, not a statistical, decision. We are satisfied that RS readings represent a valid measure of MP because of the acceptable correlation between these readings and those of HFP. Further, all relationships between MP and age, gender, and serum levels of L and Z, whether significant or insignificant, were parallel for both instruments. However, we believe that investigators should use only one instrument type for the duration of a single research project because of differences in the underlying principles of the two techniques and factors that may influence the readings.

In this study, the coefficient of variation of MP readings using RS, within a single session, matched the findings in a previous study in which RS was used ($< \pm 10\%$).[20] When compared with other techniques of in vivo measurement of MP, the reproducibility of RS MP readings correlated well with the autofluorescence technique (9%–11%) and was better than that of fundus reflectometry (19%–22%) and HFP (15%–35%, depending on age, training, and experience of subjects).[16]

Furthermore, the agreement of RS measurements taken during two separate sessions was excellent, as reflected in the Bland-Altman plots. In other words, RS measurements of MP are reproducible within a single session and are not subject to meaningful test–retest (intersessional) variability.

In our study, 4% of subjects were unable to perform HFP despite training; this has also been the experience of other investigators (5%).[43] Of note, MP readings using RS were recorded in these same subjects without difficulty.

In conclusion, RS is an objective method of measuring MP levels in healthy subjects, with acceptable reproducibility and test–retest variability. We have demonstrated good correlation between RS and HFP readings of MP and between their respective relationships with other and relevant variables such as age, gender, and serum concentrations of L and Z, thus authenticating RS against a validated psychophysical technique of measuring MP.

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