Identification of 3-Methoxyzeaxanthin as a Novel Age-Related Carotenoid Metabolite in the Human Macula

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Purpose. The xanthophyll carotenoids lutein and zeaxanthin, along with their major metabolites, *meso*-zeaxanthin, and 3′-oxolutein, are highly concentrated in the human macula. In addition to these two metabolites, there are still others that have not yet been identified. A highly sensitive HPLC-mass spectral method was used to identify and quantify a new xanthophyll metabolite that increases with age.

Methods. Maculae (4-mm diameter) from donor eyes free of ocular disease were procured from the local eye bank. The carotenoid extracts from each tissue sample were analyzed by HPLC coupled with an in-line single quadrupole mass spectrometer in a positive ion atmospheric pressure chemical ionization mode. The elution profile, visible absorption spectra and mass spectra were compared to synthetic standards to identify the ocular carotenoids and their metabolites.

Results. Along with 3′-oxolutein and *meso*-zeaxanthin, a relatively nonpolar zeaxanthin derivative was identified, with *m/z* 582.5 and spectral properties similar to those of dietary zeaxanthin. This compound was identified as 3-methoxyzeaxanthin (3-MZ) based on elution profile, absorption spectra, and mass spectra in comparison to a synthetic standard. 3-MZ increased with age ($P < 0.001$) and was not detectable in peripheral retina or in nonretinal tissues.

Conclusions. Identification of 3-MZ in the macula of aged donors indicates that O-methylation of carotenoids is a potential biomarker for aging and age-related ocular disorders. (*Invest Ophthalmol Vis Sci.* 2007;48:1435–1440) DOI:10.1167/iovs.06-1046

Age-related macular degeneration (AMD) is the major cause of registered blindness in the developed world, yet its pathogenesis remains inadequately understood. Cellular damage due to high levels of oxidative stress could be one of the main pathologic explanations for age-related diseases, including AMD.[1–3] Highly reactive molecules produced within cellular systems can initiate cytotoxic reactions that alter the structure and function of lipid, protein, and DNA within the cell with devastating results. The retina is particularly susceptible to oxidative stress due to its high oxygen use and exposure to intense light conditions. Retinal constituents such as photoreceptor outer segments, by virtue of their high polyunsaturated fatty acid content, are vulnerable to cellular damage caused by reactive oxygen intermediates.[4]

Several antioxidants such as vitamins and carotenoids are known to offer a protective mantle against these damaging molecules, potentially ameliorating diseases associated with aging, including cancer, cardiovascular disease, cataracts, and AMD. Ocular xanthophyll carotenoids such as dietary (3*R*,3′*R*)-zeaxanthin predominate in the foveal center, whereas dietary (3*R*,3′*R*,6′*R*)-lutein is more abundant farther out in the periphery.[5,6] Loss of these pigments across all areas of the retina in AMD donor eyes in comparison with control eyes supports the protective role of these pigments in the eye.[7] Carotenoids found in the retina are believed to limit retinal oxidative damage by absorbing incoming blue light and/or by quenching reactive oxygen intermediates.[1,8–10]

The physiological requirements for the xanthophyll carotenoids can be met only by dietary intake, and their antioxidant and other physiological functions generate several nondietary metabolites which have previously been characterized in the human and monkey retina. HPLC analysis of carotenoids in human ocular tissues has revealed that dietary lutein and zeaxanthin are metabolized to (3*R*,3′*S-meso*)-zeaxanthin (*meso*-zeaxanthin), (3*R*,3′*S*,6′*R*)-lutein (3′-epilutein), and 3-hydroxy-β,ε-carotene-3′-one (3′-oxolutein).[11–12] Although double-bond isomerization of lutein is believed to be the major source of *meso*-zeaxanthin, allylic oxidation of dietary lutein and/or zeaxanthin probably results in the formation of 3′-oxolutein, although the actual biochemical mechanisms remain to be defined. 3′-Epilutein and possibly *meso*-zeaxanthin may be formed as products of salvage pathways that reduce oxidized ocular carotenoids such as 3′-oxolutein. Thus, high levels of these xanthophyll carotenoid metabolites could be biomarkers of oxidative stress in the retina.

Previously published quantitative studies of ocular xanthophyll metabolites have generally used whole retinas or pooled macular tissues to achieve sufficient material to identify and measure their levels reliably. Recent improvements in in-line HPLC photodiode array and mass spectral detectors have improved detection sensitivities by a factor of ~100.[13] We used these improved techniques on individual maculae from a large number of human donor eyes, to detect novel xanthophyll metabolites and to study age-related changes in them.

Methods

Chemicals

Standards of (3*R*,3′*R*,6′*R*)-lutein, (3*R*,3′*S*)-*meso*-zeaxanthin, (3*R*,3′*R*)-zeaxanthin, and 3′-oxolutein were generous gifts from Kemin Health

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TABLE 1. Macular Carotenoids Separable on Silica Nitrile and C30 Columns

Peak	Retention Time (min)	Wavelength* (nm)	Major Mass Peak (*m/z*)	Carotenoids
Main Peaks Separated on Silica Nitrile Column				
1	5.12	423/**452**/478	582.5	3-Methoxyzeaxanthin
2	7.69	427/**447**/476	566.5	3′-Oxolutein
3	7.92	417/**441**/470	549.5	Lactucaxanthin
4	8.60	424/**447**/475	568.5	(3*R*,3′*R*,6′*R*)-Lutein
5	9.46	424/**453**/479	568.5	Mixture of (3*R*,3′*R*)-zeaxanthin and (3*R*,3′*S- meso*)-zeaxanthin
6	9.97	421/**442**/465	568.5	(3*R*,3′*S*,6′*R*)-Lutein (3′-epilutein)
7	10.91	427/**448**/475	568.5	9-*cis*-Lutein
8	11.67	421/**445**/471	568.5	9′-*cis*-Lutein
9	13.13	421/**453**/476	568.5	9-*cis*-Zeaxanthin
Main Peaks Separated on C30 Column				
1	10.88	416/**440**/466	568.5	13- or13′-*cis*-Lutein
2	11.82	328/412/**439**/466	568.5	*cis*-Lutein
3	12.44	428/**446**/474	566.5	3′-Oxolutein
4	13.56	424/**446**/474	551.5†	(3*R*,3′*R*,6′*R*)-Lutein
5	14.98	424/**446**/466	540.5	Unidentified
6	15.94	427/**451**/479	568.5	Mixture of (3*R*,3′*R*)-zeaxanthin and (3*R*,3′*S-meso*)-zeaxanthin
7	19.47	332/418/**446**/474	564.5	Unidentified
8	21.57	422/**452**/474	582.5	3-Methoxyzeaxanthin

* Absorbance maxima data in bold indicate main peak of the spectrum.

† (3*R*,3′*R*,6′*R*)-Lutein undergoes loss of a water molecule under these chromatographic and ionization conditions.

(Des Moines, IA), DSM (Schaffhausen, Switzerland), and BASF (Ludwigshafen, Germany). They were individually dissolved in hexane at concentrations of 1 μg/mL and dried and stored at −70°C. Composite working standard solutions were prepared by combining suitable aliquots of each individual standard stock solution and diluting them with HPLC mobile phase. Organic solvents were HPLC-grade from Fisher Scientific (Hampton, NH).

Preparation of Macular and Retinal Samples

Human donor human eyes were obtained from the Utah Lions Eye Bank within 24 hours after death after corneas had been harvested for transplantation. Tissue procurement and distribution complied with the tenets of the Declaration of Helsinki. All eye cups were visually inspected with a handheld magnifier to exclude the presence of obvi-

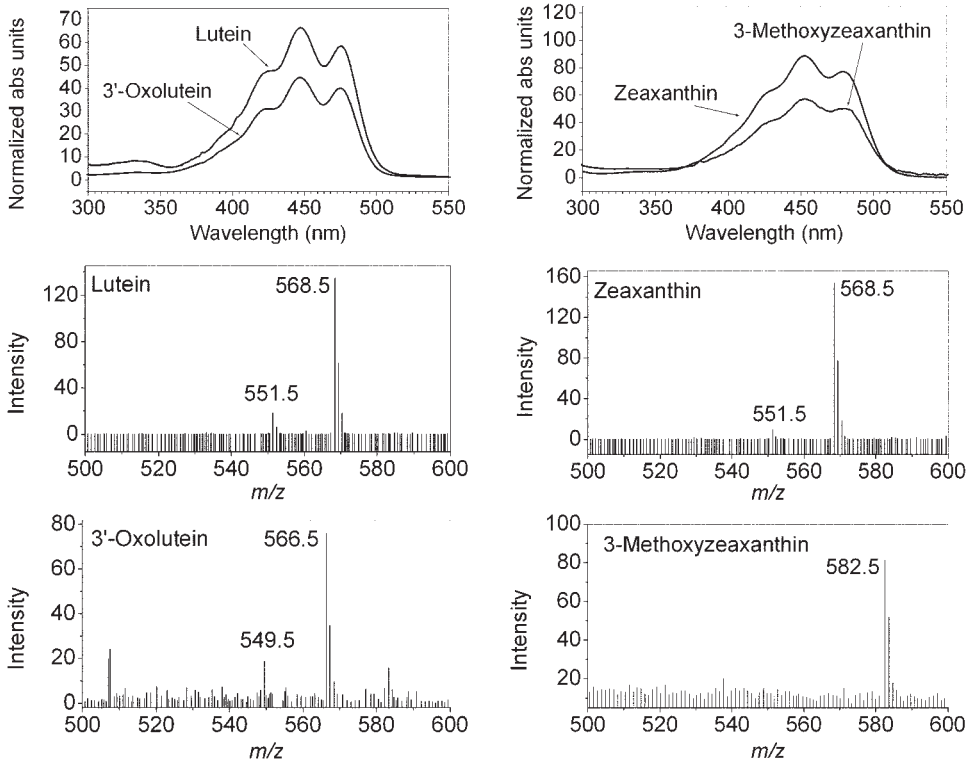

FIGURE 1. Absorption and mass spectra of lutein and zeaxanthin and their metabolites isolated and purified from human macula.

ous ocular disease such as disciform scars, choroidal neovascularization, geographic atrophy, or large drusen. After overlying vitreous was carefully removed, macular tissue was excised with a 4-mm diameter circular trephine centered on the fovea, and peripheral equatorial retinal tissue was excised with an 8-mm diameter circular trephine making certain that the nearest edge of the punch was at least 10 to 12 mm away from the fovea. Tissue samples were extracted three times with tetrahydrofuran (THF) containing 0.1% butylated hydroxytoluene (BHT) by sonication at 5°C to 10°C for 30 minutes each time. The combined extracts were evaporated to dryness under vacuum at room temperature. The residue was redissolved in the HPLC mobile phase, and the vials were centrifuged at approximately 2000*g* to remove minor amounts of insoluble solid particles. Dichloromethane, chloroform, and methanol were not used for extraction to avoid artifactual methylation of carotenoids, and the THF/BHT solution was freshly prepared from small, sealed bottles to minimize peroxide formation. Samples were stored at −70°C until analyzed.

HPLC Conditions

The samples were analyzed on three HPLC systems on a gradient HPLC apparatus (Thermo Separations; San Jose CA) equipped with a high-sensitivity UV6000 photodiode array detector. System 1: Mobile phase containing hexane-dichloromethane-methanol: *N*,*N*′-di-isopropylethylamine (80:19.2:0.7:0.1 vol/vol); HPLC separation was performed at a flow rate of 1.0 mL · min^{-1} on a cyano column (Microsorb 25 cm length × 4.6 mm inside diameter [ID]; Rainin Instrument Co., Woburn, MA). System 2: mobile phase containing methanol and dichloromethane; HPLC separation was performed at a flow rate of 1.0 mL · min^{-1} on a C30 column (Waters 25 cm length × 4.6 mm ID; Waters, Milford, MA) by a linear gradient of methanol and methylene chloride. A mobile phase of methanol (100%; phase A) and methylene chloride (100%; phase B) with the following linear gradient elution was developed: 90% A and 10% B in the beginning, maintained for 5 minutes, decreased to 78% A in 15 minutes, 62% A in 30 minutes, 52% A in 40 minutes, 41% A in 50 minutes, and 38% A in 58 minutes and returned to 90% A in 60 minutes. System 3: Mobile phase containing hexane: iso-propanol (95:5 vol/vol); HPLC separation was performed at a flow rate of 0.7 mL · min^{-1} on a chiral column (ChiralPak, 25 cm length × 4.6 mm ID; Chiral Technologies, Exton, PA). All columns were maintained at room temperature, and the HPLC detector was operated at 450 nm. Peak identities were confirmed by photodiode-array (PDA) spectra and by co-elution with authentic standards as necessary. We do not routinely include an internal standard because it can mask the presence of low-abundance carotenoid metabolites, especially when samples are split and run on multiple HPLC columns. We periodically perform quality control runs to confirm that our extraction and injection efficiencies exceed 95%.

Mass Spectrometry Equipment and Conditions

MS analysis was performed with a single quadrupole mass spectrometer (MSQ; Thermo Electron), equipped with an atmospheric pressure chemical ionization (APCI) source after 50% of the PDA eluate had been directed to waste with the help of a diverter valve. Both full-scan (FS) and single-ion-monitoring (SIM) modes were used. The positive molecular ions were initially acquired in full-scan mode from 200 to 1000 Da with 0.2-step size and 2-ms dwell time. SIM was performed with a dwell time of 200 ms for each channel. SIM mode target ions were *m/z* 551 ±1.5 and 569 ±1.5 for lutein, 569 ±1.5 for zeaxanthin, and 567 ±1.5 for 3′-oxolutein. 3′-Epilutein was present in only trace amounts. Extracted ion chromatograms yielded an unexpected peak arising from an ion at 582.5 *m/z,* and thus SIM chromatograms at 582.5 ±1.5 *m/z* were also analyzed. Ion chromatograms were generated by monitoring these ions, which were also used for quantification by external standardization with authentic standards. Typical conditions were corona discharge current, 5 μA; RF lens bias voltage, 0.1 V; cone voltage, 80 V; and heater temperature, 550°C. The ion source and tuning lens parameters were optimized automatically by infusing standards via the built-in injector. Routine quantitative analysis was performed on a commercial system (MSQ Quan Browser, Xcaliber; ThemoElectron) was used. The software provides a peak integration of the ion intensity for each of the programmed mass ranges. Automated

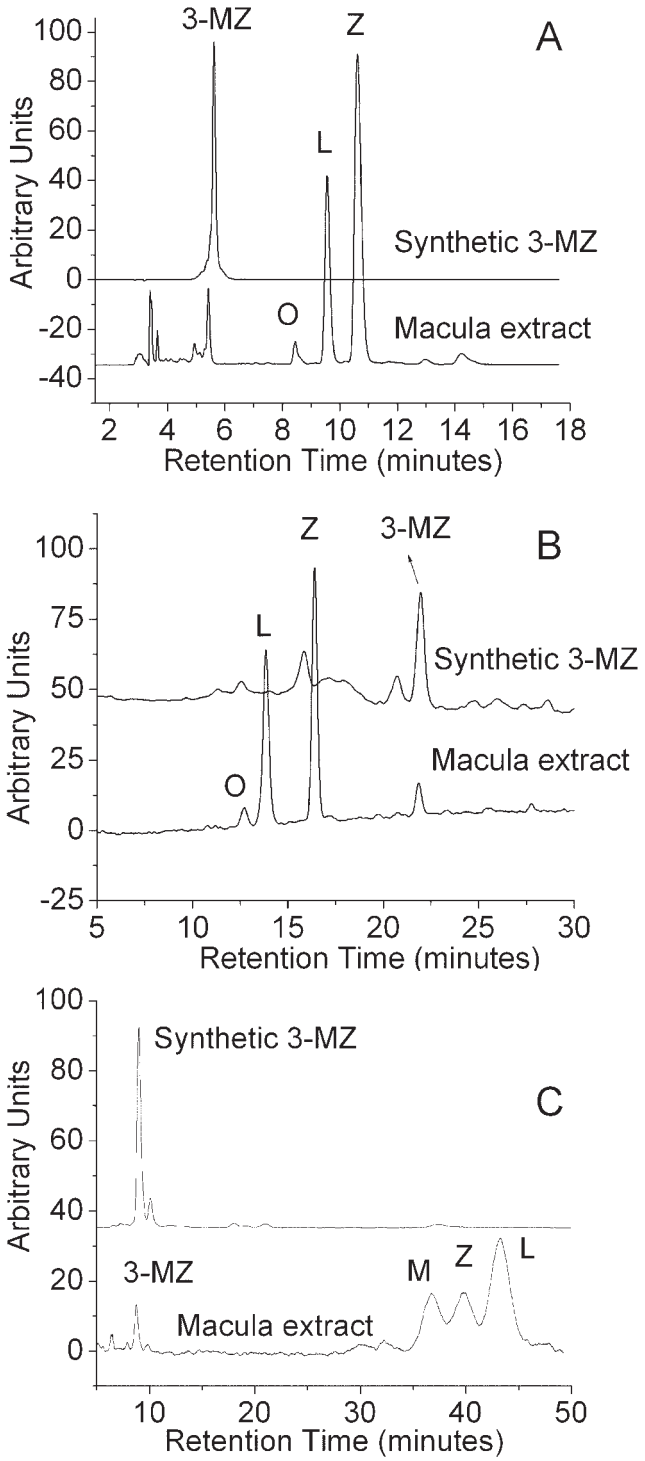

Figure 2. HPLC chromatograms of carotenoids extracted from the macula of a 73-year-old donor in comparison to synthetic 3-MZ. Analysis was performed on a silica nitrile column (**A**), a C30 column (**B**), and a chiral column (**C**). Major carotenoids found in the macula were (3*R*,3′*R*,6′*R*)-lutein (L), (3*R*,3′*R*)-zeaxanthin (Z), (3*R*,3′*S-meso*)-zeaxanthin (M), 3-methoxyzeaxanthin (3-MZ), and 3′-oxolutein (O). Z and M co-eluted on columns A and B.

TABLE 2. Macular Carotenoid Content of a Normal Population

	Young Age (<48 y)	Old age (≥48 y)	Change
Average age (y)	32 ± 8 (n = 35)	68 ± 7 (n = 38)	
Carotenoids (ng)			
Total carotenoids	22.0 ± 9.6	25.8 ± 12.9	17% ↑ (P = 0.33)
(3*R*,3′*R*,6′*R*)-Lutein	9.4 ± 4.2	9.8 ± 8.0	4% ↑ (P = 0.42)
(3*R*,3′*R*)-Zeaxanthin	6.7 ± 3.6	7.5 ± 5.5	12% ↑ (P = 0.40)
(3*R*,3′*S-meso*)-Zeaxanthin	3.7 ± 5.4	8.2 ± 6.7	122% ↑ (P < 0.001)
3′-Oxolutein	0.6 ± 0.5	1.3 ± 1.8	117% ↑ (P < 0.001)
3-MZ	1.4 ± 1.1	3.8 ± 2.8	172% ↑ (P < 0.001)

analysis was followed by manual confirmation of in-source fragmentation patterns.

For calibration curves, standard solutions were injected in different volumes, so as to achieve final injected amounts ranging from 1 to 1000 pg for mass spectral measurements and 1 to 8 ng for PDA measurements. Accuracy and precision of the methods were determined by generating intra- and interday variability data from a series of samples in the range of 1 to 1000 pg injected five times on a single day. Limits of detection and limits of quantitation were determined by preparing standard curves with three concentrations, 1.0, 5.0, and 8.0 ng/mL each of dietary zeaxanthin and lutein. Analysis was performed in triplicate, and the calibration curves were obtained by plotting concentration against peak area.

Synthesis of 3-Methoxyzeaxanthin

3-Methoxyzeaxanthin (3-MZ) was prepared as described by Mueller and Karrer in 1965.[14] Zeaxanthin (0.5 g) was dissolved in 3 mL dimethylsulfoxide and 2 mL dimethylformamide. Methyl iodide (3 mL) and barium oxide (6 g) were added and stirred overnight at 35°C. Then, another 5 mL of methyl iodide and 6 g of barium oxide were added, and stirred at 35°C for another 24 hours. The contents of the flask were extracted four times with 50 mL of chloroform each time. The chloroform extracts containing the product were washed four times with water and then dried with sodium sulfate. The chloroform was removed under vacuum, and the residue was dissolved in HPLC mobile phase (system 2) and purified by HPLC and fraction collection. The identity of the purified fraction was confirmed by mass spectrometry and by absorption spectroscopy.

Statistics

Statistical analysis was performed with commercial software (Origin ver. 6.0; Microcal, Northampton, MA). In most cases, a two-population (independent) t-test was performed with significance level set at 0.05. All reported results are expressed as the mean ± SD.

RESULTS

For routine analysis of carotenoids in human maculae, our laboratory usually uses a normal-phase silica-based nitrile column because it has short run times, and lutein and zeaxanthin are well separated. Nine carotenoids are consistently resolved from human macula extracts on this column. The major molecular ions and visible absorption properties of these nine carotenoids are shown in Table 1. Eight of these nine carotenoids had been identified in primate macular extracts, but the well-resolved peak at approximately 5.0 minutes had not. This carotenoid had a major m/z of 582.5 compared with lutein's and zeaxanthin's m/z of 568.5, yet it had a visible absorbance identical with (3*R*,3′*R*)-zeaxanthin.

This unidentified peak also had a minor m/z of 564.5 and absorbance in the 320- to 350-nm range of variable intensities which suggested the presence of a co-eluting UV-active contaminant. Therefore, we reanalyzed macular extracts on a reversed-phase C30 column. Eight carotenoid peaks are resolvable on this column, and their masses and visible absorption properties are shown in Table 1. Peak 8 on the C30 column had identical major mass and visible absorption properties as peak 1 on the nitrile column, but it no longer had any UV absorbance or m/z of 564.5, suggesting that the co-eluting contaminant seen on the nitrile column was no longer present. To prove the identity of nitrile peak 1 and C30 peak 8, we collected peak 1 from the silica nitrile column and then reran it on the C30 column where it eluted with a primary peak retention time and m/z identical with C30 peak 8, and the minor contaminant eluted with a retention time and m/z identical with C30 peak 7.

The identical absorption spectrum to that of zeaxanthin and the increased mass of 14 Da suggested that this unidentified carotenoid may represent O-methylated zeaxanthin. We therefore synthesized 3-MZ by previously published methods[14] from both (3*R*,3′*R*)-zeaxanthin and (3*R*,3′*S-meso*)-zeaxanthin, and we confirmed that their m/z was 582.5 on our mass spectrometer and that their visible absorption spectra matched unmethylated zeaxanthin (Fig. 1). Co-elution of nitrile peak 1 and C30 peak 8 with synthetic 3-MZ confirmed that the previously unknown macular carotenoid was 3-MZ (Figs. 2A, 2B). We further studied 3-MZ on a chiral column known to separate dietary zeaxanthin from *meso*-zeaxanthin to see whether we could resolve 3-MZ made from (3*R*,3′*R*)-zeaxanthin from 3-MZ made from (3*R*,3′*S-meso*)-zeaxanthin. Using our standard chiral HPLC analytical conditions, the synthetic and naturally formed 3-MZ all elute at approximately 9 minutes (Fig. 2C). Since we have not yet been able to separate the two forms of synthetic 3-MZ by chiral HPLC, we are unable to comment on the precursor to macular 3-MZ. We also synthesized 3-methoxylutein and the dimethyl ethers of lutein and zeaxanthin and found them in no more than trace amounts in any macula extracts.

In the peripheral retina where carotenoid concentrations are at least 10-fold lower than in the macula, 3-MZ was undetectable, even when 8-mm diameter punches were examined, nor could we detect it in human lens, serum, or liver. 3-MZ was not present in the zeaxanthin-rich Japanese quail retina, but this is not surprising, because most of its ocular zeaxanthin is esterified and therefore unavailable for O-methylation. We also noted in our initial studies of 3-MZ that macular levels were typically much lower in younger eyes. We therefore systematically re-examined all available chromatograms from a previous study we conducted in which we measured macular carotenoids from 228 eyes from 147 donors with a wide range of ages (5–86 years) to determine whether or not 3-MZ truly rises with age.[15] We first excluded 29 outlier eyes that had unusually high levels of total macular carotenoids (>50 ng in a 4-mm punch) that could skew the data. All the outliers were age 48 or older, and most had been consuming high-dose lutein supplements regularly before death.[15] Chromatograms from 73 nonout-

lier eyes were available for reanalysis (Table 2). Figure 3 shows that even after excluding the older outliers, macular 3-MZ rose significantly with age ($P < 0.001$). In this set of eyes, dietary lutein and zeaxanthin do not rise significantly with age; however, their major metabolites, 3-MZ, 3′-oxolutein, and *meso*-zeaxanthin, all increase significantly with age ($P < 0.001$; Table 2).

Discussion

In this study, we definitively demonstrated by mass spectrometry, absorption spectroscopy, and HPLC co-elution with synthetic standards that 3-MZ is a major xanthophyll carotenoid metabolite in the human macula. Some previous HPLC studies of human maculae have missed this compound because they used internal standards such as the O-methyl and O-ethyl ethers of lutein, which would be expected to mask its presence.[11,16–17] To our knowledge, this is the first report of carotenoid O-methylation in any nonplant eukaryotic system. 3-MZ is apparently macula specific, implying that it is formed there. Moreover, its levels increase substantially with age in a manner paralleling age-related DNA hypermethylation.[18] Although carotenoid O-methylation is uncommon, it has been reported in a few biological systems such as *Rhodobacter capsulatus* which possesses an enzyme that can O-methylate 1-hydroxy carotenoids in the spheroidene/1′-HO-spheroidene pathway in an S-adenosylmethionine-dependent manner.[19]

The physiological significance and biochemical origin of 3-MZ is still uncertain. It is possible that 3-MZ is formed by direct O-methylation of (3*R*,3′*R*)-zeaxanthin or (3*R*,3′*S-meso*)-zeaxanthin (Fig. 4), but the lack of significant amounts of 3-methoxylutein or any xanthophyll dimethyl ethers is puzzling if this is truly an important synthetic pathway. Alternatively, 3-MZ may be formed as the result of salvage pathways that perform concerted or sequential reductive O-methylation of oxidized xanthophylls in the macula. A proposed pathway using the most prevalent oxidized carotenoid in the retina, 3′-oxolutein, as the precursor is shown in Figure 4. The fact that 3-MZ accumulates with age supports this hypothesis. Future studies of the correlation of macular 3-MZ with other biomarkers of oxidative stress in normal and AMD eyes should provide additional insights into the pathogenesis of this blinding disorder.

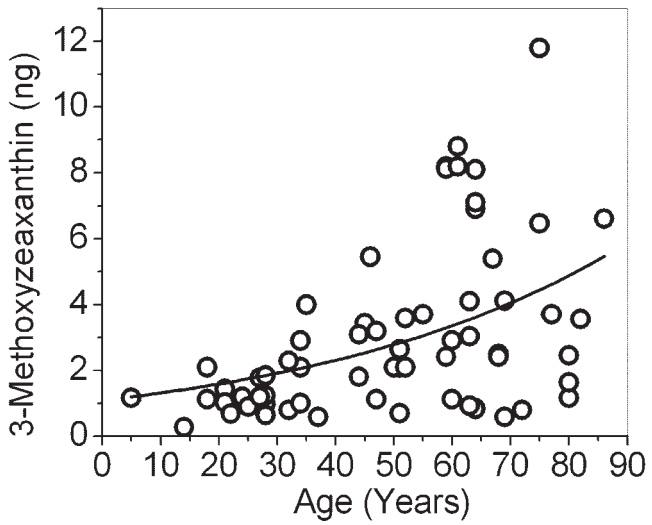

Figure 3. Age-wise distribution of 3-MZ in normal eyes. Carotenoids were extracted from 4-mm macular punches ($n = 73$) and analyzed by HPLC-APCI/MS.

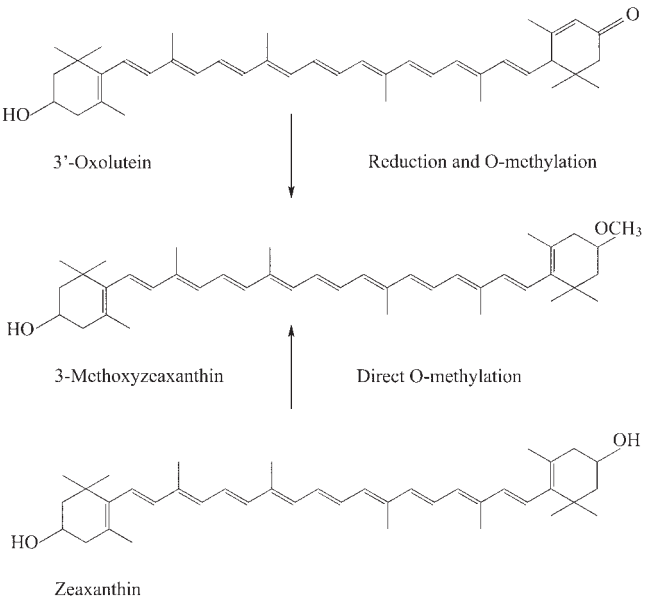

Figure 4. Structures and possible chemical conversions of zeaxanthin and 3′-oxolutein to 3-MZ.

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