

What is meso-zeaxanthin, and where does it come from?

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Abstract

The carotenoids lutein (L), zeaxanthin (Z), and meso-zeaxanthin (MZ) accumulate in the central retina, where they are collectively known as macular pigment (MP). Each of these three compounds exhibit a regional dominance, with MZ, Z, and L being the dominant carotenoids at the epicentre, mid-periphery, and periphery of the macula, respectively. There is a growing and evidence-based consensus that MP is important for optimal visual performance, because of its blue light-filtering properties and consequential attenuation of chromatic aberration, veiling luminance, and blue haze. It has also been hypothesised that MP may protect against age-related macular degeneration because of the same optical properties and also because of the antioxidant capacity of the three macular carotenoids. Challenges inherent in the separation and quantification of MZ have resulted in a paucity of data on the content of this carotenoid in foodstuffs, and have rendered the study of tissue concentrations of this compound problematic. As a consequence, the few studies that have investigated MZ have, perhaps, been disproportionately influential in the ongoing debate about the origins of this macular carotenoid. Certainly, the narrative that retinal MZ is derived wholly and solely from retinal L needs to be revisited.

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The macular carotenoids and their functions

The central retina, known as the macula, is responsible for optimal spatial vision.¹ A yellow pigment composed of the carotenoids meso-zeaxanthin (MZ), lutein (L), and

zeaxanthin (Z) accumulates at the macula where it is known as macular pigment (MP).²

The anatomical (central retinal),² biochemical (antioxidant),³ and optical (short-wavelength filtering)⁴ properties of MP have generated interest in the role of MP for vision and macular health. In the first instance, there is an evidence-based consensus that MP is important for vision in normal subjects,^{5–7} which rests on MP's ability to optimise visual performance and experience by attenuating chromatic aberration, veiling luminance, and blue haze.⁸

In the second instance, MP has generated interest because of its possible protective role for age-related macular degeneration (AMD), the world's leading cause of age-related blindness.⁸ The protection that MP may offer patients afflicted with, or at risk of, AMD is putatively attributable to its antioxidant properties^{9,10} and/or its pre-receptor filtration of damaging (short-wavelength) blue light.

Retinal anatomy of the macular carotenoids

MP is at its highest concentration in the receptor axon layer and in the inner plexiform layers, and the concentration of MP peaks at the foveola.^{11,12} L is the dominant carotenoid in the peripheral macula, Z in the mid-peripheral macula, and MZ at the epicentre of the macula.² A thorough understanding of the mechanisms governing the selective uptake of MP by the retina has proven to be elusive, although xanthophyll-binding proteins have been identified for L¹³ and Z,¹⁴ and MZ is also known to bind to a Z-binding protein¹⁵ (study into the nature of the MZ-binding protein is currently underway).¹⁴ Importantly, it has been shown that the Z-binding protein has equal affinity for MZ.¹⁵

Chemical structure of the macular carotenoids

The term carotenoid is used to describe a family of natural organic compounds build from an

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assembly of isoprenoid units that are important in many aspects of life. Because of their mostly coloured nature and consequential ability to absorb visible light, carotenoids are often referred to as pigments or chromophores.¹⁶ All carotenoids have a characteristic linear conjugated polyene chain and are classed into one of two subgroups. The hydrocarbon carotenoids are known as carotenes, such as β -carotene and lycopene, whereas oxygenated derivatives are called xanthophylls, or oxy-carotenoids, and include MZ, L, and Z (Figure 1).¹⁶

The xanthophyll macular carotenoids consist of the typical C40 carotenoid structure, and are substituted with hydroxyl groups at the 3 and 3' carbon positions. Z and MZ both contain two β -ring end groups, whereas L contains both a β -ring and an ϵ -ring, thereby reducing the level of the conjugation chain length observed in L. In addition, L has three chiral positions and is therefore described as 3R,3'R,6'R L. Z and MZ are classed as diastereomers, and differ only in the spatial orientation of the hydroxyl group on the C3' chiral position. This chiral position within MZ has an S spatial orientation, whereas Z has an R spatial orientation. This difference in spatial arrangement results in MZ being described as 3R,3'S Z, and Z being described as 3R,3'R Z. These structural differences of MZ, L, and Z have important implications for their respective antioxidant and light-filtering properties.^{10,17}

Chemical, light-filtering, and antioxidant properties of the macular carotenoids

The characteristic vibrant hues of carotenoids are a result of light-absorbing properties provided by their inherent

conjugated double-bond system. The resultant UV/vis absorption spectra are routinely used to assist in the identification and characterisation of carotenoids, and have been extensively studied.^{18,19} The absorption spectra of MP peaks at 460 nm.²⁰ Although MZ, L, and Z exhibit very similar absorption spectra, it is possible to distinguish them from one another based on slight variations in relative absorbances (nm) and intensities (AU).²¹

At the macula, L is reported to be a superior filter of short-wavelength (blue) light when compared with Z, because of its orientation with respect to the plane of the phospholipid bilayer of the cell membrane, which is both parallel and perpendicular.²² In contrast, Z and MZ exhibit an orientation only perpendicular to this layer. However, structural differences and the consequential differing absorption spectra of these pigments (MZ, L, and Z) result in a collective optimal filtration of blue light at the macula, which would not be achieved by any of these carotenoids in isolation.²³

The polyene chains of MZ, L, and Z supply readily available electrons that enable these carotenoids to quench reactive oxygen species (ROS), thus limiting membrane phospholipid peroxidation and attenuating oxidative injury.^{16,17,22,24,25} Z is twice as efficacious as L at quenching ROS, and this is attributed to the extended conjugated system of Z in comparison with L.²⁶ This extended conjugation is also present in MZ. When in conjunction with a Z-binding protein, MZ has proven to be a more potent antioxidant than Z.¹⁴ Of interest, a mixture of L, Z, and MZ *in vitro*, in a ratio of 1:1:1, has been shown to quench more singlet oxygen than any of these individual carotenoids at the same total concentration.¹⁰

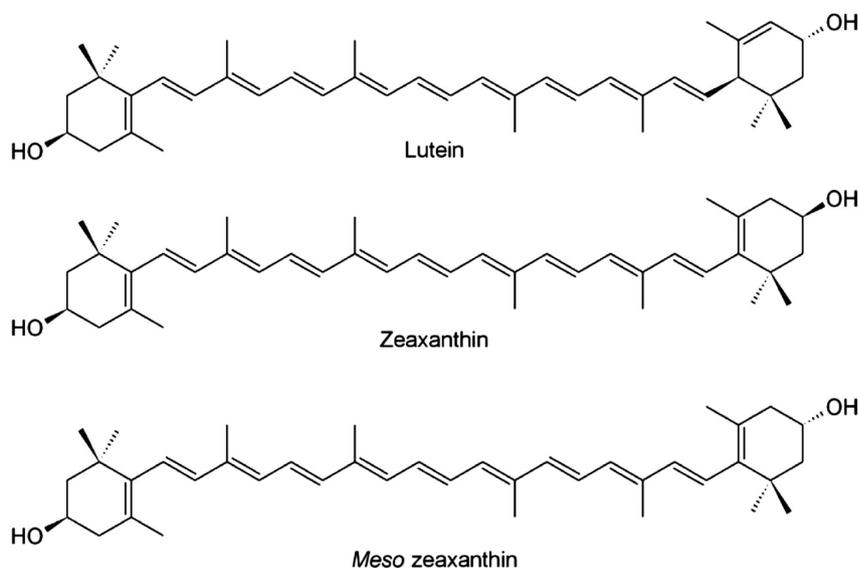


Figure 1 Chemical structures of the three macular carotenoids L, Z, and MZ.

Identification and quantification of the macular carotenoids

To quantify MZ, L, and Z, current methodology requires two successive HPLC separations (assays). The first assay (reverse phase chromatography) separates L from a total Z fraction, which contains Z and MZ. This total Z fraction is typically manually collected, and then analysed using the second assay (normal phase chromatography). This second assay is required to separate Z and MZ, so that quantification of these individual carotenoids can be achieved. This general method of carotenoid analysis has been adapted and modified by several laboratories for the purpose of MZ quantification.^{21,27–30} Modifications are typically made to the first assay, where a variety of reverse phase columns and solvent compositions can produce acceptable L and total Z separations.³¹ However, the normal phase column (Daicel ChiralPak AD (Daicel Chiral Technologies Europe, Illkirch – Cedex, France); 250 × 4.6 mm²) used in the second assay has remained constant and universal among studies reporting on MZ.

Although the above two-step method is successful at quantifying MZ, it is limited in a number of ways. First, the method is labour intensive because of the requirement to manually collect the total Z fraction. Also, manual collection is operator-dependent, and therefore vulnerable to human error. As a result, only a portion of the total Z fraction is collected, and is typically contaminated with L carryover. Second, the relatively long time for sample analysis (in some cases, as much as two hours per sample) makes bulk analysis difficult and costly. Recent developments by our laboratory have included automation of the fraction collection step, which has greatly reduced operator requirements and study analysis time, in addition to improvements in fraction recoveries and elimination of L carryover. These refinements to the first assay, in combination with advances in HPLC technologies and analytical methodologies, have resulted in better resolution and greater sensitivity for MZ separation and quantification.

Origin of the macular carotenoids

To date, approximately 700 carotenoids have been isolated and identified in nature, with in excess of 40 found in fruits and vegetables.^{32,33} Despite this, only 14 of these dietary carotenoids may be absorbed, modified, and/or used by the human body,³⁴ and yet only MZ, L, and Z are found at the macula, reflecting an exquisite degree of biological selectivity, which is unlikely to be accidental in design.³⁵

Studies have shown that L and Z are plentiful in many foodstuffs typical of a western diet (eg, spinach, kale,

corn, and egg products).³⁶ However, there is a paucity of research investigating MZ in foodstuffs, apart from a report by Maoka *et al*³⁷ in 1986, where MZ was confirmed to be present in 21 species of edible fish, shrimp, and sea turtles, and a recent report by Rasmussen *et al*²¹ who reported the presence of MZ in Californian and Mexican hen eggs. Of note, the Mexican hens were given MZ-enriched feed. However, in contrast to the Maoka publication, Rasmussen *et al*²¹ did not detect the presence of MZ, L, or Z in any fish (eg, haddock and sea bass) or seafoods (eg, shrimp) analysed in their study. Importantly, there is a substantial discrepancy between the extraction methods used by Rasmussen *et al* vs those used by Maoka *et al*, which merits discussion. Mainly, Rasmussen *et al* did not saponify the foods before analysis. Saponification is an alkaline hydrolysis (ie, breakdown) of the ester linkages between the carotenoids and lipid (fatty acid) molecules. This is a required step in the process of carotenoid extraction, as it frees esterified carotenoids in the food sample that otherwise would not be observed during analysis, thereby resulting in an underestimation of MZ. Indeed, this point is consistent with and corroborated by Rasmussen *et al*'s own findings, as they identified all three macular carotenoids in egg yolk, because egg carotenoids are not esterified (and therefore the need for saponification is precluded).

It is surprising, therefore, that the view that MZ is 'non-dietary' in origin has prevailed and gone largely uncontested, given that the MZ content of foodstuffs that contain carotenoids (eg, corn, orange peppers, and yellowtail tuna) has been neither extensively nor satisfactorily investigated.

It had been suggested that the MZ found and reported by Maoka *et al*³⁷ was just an artefact of degradation from L,²¹ which occurred during the sample-extraction saponification process (given that the biotransformation of L to Z is an alkaline hydrolysis reaction).³⁵ However, we have tested this possibility in our laboratory and found that saponification of purified L does not generate MZ, even with high concentrations of KOH. Importantly, for this experiment we purified our L standard via HPLC, as previous work by our laboratory indicated the presence of all Z isomers (including MZ) in DSM-provided L standards.³⁸ Of note, it has also been suggested that MZ may be generated from L as a result of an enzymatic process at the macula, but such a hypothesis has yet to be investigated.²⁸

Also, and of interest, a recent investigation of serum carotenoid response following supplementation with different macular carotenoid formulations identified an unexpected peak with the spectrophotometric characteristics of MZ in the serum of subjects supplemented with 20 mg of L and 2 mg of Z (Ultra Lutein from Natural Organics Inc., Melville, NY, USA:

L provided by FloraGLO, which is a registered trademark of Kemin Health, L.C., Kemin Industries, Inc. Des Moines, IA, USA). Based on label claim, these subjects received no MZ. Given that this was an unexpected finding, we reanalysed random serum samples from these subjects in order to confirm or refute our observation, and confirmed that MZ was, indeed, present.³⁸ We then tested the intervention formulation, and determined that it did, in fact, contain MZ (0.3 mg/capsule) (Figure 2), although MZ was not claimed on the product label.

These findings have implications for past and ongoing research surrounding carotenoid supplementation. Indeed, any discrepancy between actual and alleged concentrations of the respective macular carotenoids in commercially available preparations is important when such formulations are used for the purpose of research, particularly clinical trials. Study on the actual *vs* declared carotenoid content of commercially available supplements is currently being investigated by our group.

Further, our finding that some commercially available L supplements contain MZ, but where the presence of MZ is neither recognised nor acknowledged in the supplement label, has profound implications for our current understanding of the origins of MZ, as it has been proposed that retinal MZ is derived solely from retinal L. Of note, this hypothesis is largely driven by the findings of a single DSM-sponsored study, where MZ was identified in the retinae of L-supplemented carotenoid-deprived rhesus monkeys.²⁹

This influential study included four groups of six rhesus monkeys. Three groups were maintained on a xanthophyll-free diet, and one group was given a standard diet. Two of the three xanthophyll-free groups were then given xanthophyll supplements. The first were

given L only and the second were given Z only, whereas the third acted as a control (remaining xanthophyll-free). Two monkeys from each group were killed at 6–10-month intervals during the 24-month study period, and their retinae were removed and analysed for carotenoid content. As expected, the control group demonstrated a complete lack of any xanthophyll carotenoid at the macula. The investigators detected MZ and L in the central retinae of L-supplemented monkeys, but only Z in the Z-supplemented primates, and concluded that retinal MZ was derived wholly from retinal L.²⁹

However, we believe that the conclusion by Johnson *et al*²⁹ cannot and does not follow from their findings, as no study animals were supplemented with MZ, thereby precluding meaningful comment on the bioconversion-dependent interrelationships (if any) between the three macular carotenoids.

Further, the recent identification of MZ in Ultra Lutein, which contains DSM-sourced L, using novel and validated technology, lends credence to the possibility that the L-supplemented rhesus monkeys were in fact being supplemented with small amounts of MZ, thus accounting for the reported concentrations of MZ detected in the retinae of those animals. Of note, the authors state that the L supplement used in their study was purified L generated for the purpose of their research, although the method of purification was not described and therefore cannot be tested by another laboratory. In addition, it is possible that trace amounts of MZ may not have been detected in the supplement used by the methodologies available at that time. Also, and of concern, an unknown peak was found to co-elute with the Z fractions of retinal samples in the report by Johnson *et al*²⁹, which affected accurate quantification of the carotenoids present in these samples. The researchers did make some attempt to address this latter issue by using a

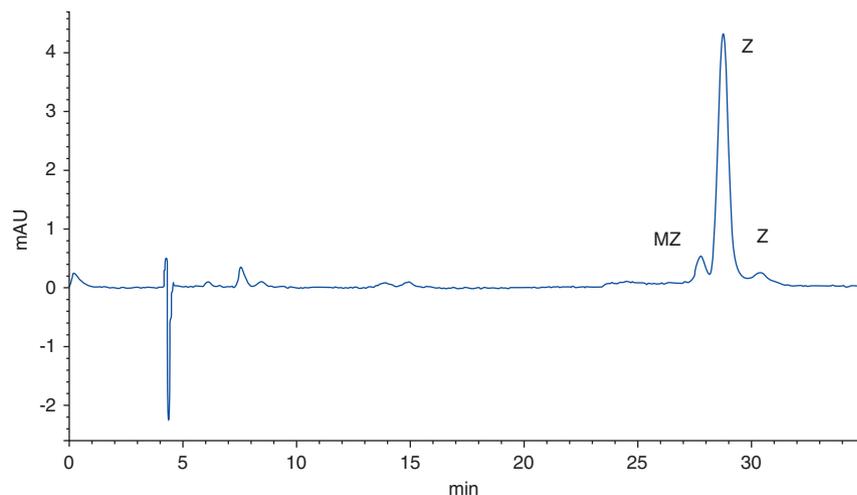


Figure 2 HPLC analysis of the Ultra Lutein supplement.

method of proportions to identify the contribution of this unknown peak to the Z fraction. Their method, however, depended on precise and total collection of both L and Z fractions (combined) from the first analysis, which, we believe, is impossible to ensure when collecting carotenoid fractions manually (as in their study). Therefore, although the publication reported the presence of MZ following supplementation, the reported quantities of MZ and Z remain questionable. It should also be noted that the researchers did attempt to identify the unknown peak, but conceded that their efforts in this regard were unsuccessful. Accordingly, and in keeping with best scientific practise, we believe that work conducted by Johnson *et al*²⁹ needs to be replicated and extended to include animals supplemented with MZ so that the benefits of recent advances in separation science can be employed in this endeavour.

The hypothesis that retinal MZ is wholly and solely the result of bioconversion of retinal L is inconsistent with the findings of Bhosale *et al*,³⁹ who measured deuterated (D)-L, D-Z, and D-MZ in the retinae of quail following supplementation with either D-L, D-Z, or regular diet (control group). After killing, D-L and D-MZ were identified only in animals supplemented with D-L, whereas D-Z was the only isotopically labelled macular carotenoid identified in animals supplemented with D-Z. However, there was a marked discrepancy in the proportion of total retinal L that was deuterated (83%) and the proportion of total retinal MZ that was deuterated (42%), suggesting that retinal MZ is not derived entirely or exclusively from retinal L.

Of course, it could be postulated that there was a lag in the conversion of supplemented L to MZ, thus explaining the observed discrepancy between the proportions of MZ and L that were deuterated, but such a hypothesis requires investigation. Moreover, no study animal was supplemented with isotopically labelled MZ, thereby precluding the opportunity to investigate the possibility of bioconversion of this carotenoid. It is also important to note that the quails were not carotenoid-deficient before supplementation, and therefore the study was not designed to test whether L is the sole precursor of MZ. Furthermore, the work by Bhosale *et al*,³⁹ should be interpreted with full appreciation of the questionable appropriateness of a non-primate, such as quail, as an animal model for the study of human MP and its origins.³¹

Safety of MZ

MZ is now available in commercially available preparations (eg, MacuShield (Macuvision Europe Ltd, Solihull, UK), MacuHealth with LMZ3 (MacuHealth LLC, Birmingham, MI, USA), and Lutein Plus (Holland

& Barrett, Nuneaton, UK)), and many have been consuming MZ in supplement form since the 1990s, and there are no published accounts of adverse events or safety issues arising from its use. Recently published trials have found that subjects supplemented with all the three macular carotenoids (MZ, L, and Z) exhibit significant increases in serum concentrations of these carotenoids and an associated augmentation in MP.^{30,40} Indeed, a recent publication reports that the typical central peak of MP can be realised in subjects with atypical MP spatial profiles at baseline when supplemented with a preparation containing all the three macular carotenoids, but not with a supplement lacking MZ.⁴¹

MZ in such supplements is derived from natural L, which has been extracted from the Aztec Marigold flower, and has been used to date in many clinical trials and subjected to toxicity studies.^{28,30,40} A recent study of supplemental MZ in human subjects has reported that renal and liver function, as well as lipid profile, haematological parameters, and markers of inflammation, are unaffected following supplementation with a formulation containing MZ, L, and Z.³⁰

MZ is also a component of a typical diet in countries and states where it is commonly used in hen feed to enhance the colouration of the egg yolk by the poultry industry (eg, Mexico), and no associated adverse events have ever been reported.

Moreover, the safety of MZ has been further evaluated in a recent toxicity trial using an animal model (Wistar rats).⁴² The results of this trial demonstrated that the NOAEL ('No Observed-Adverse-Effect Level') was in excess of 200 mg/kg/day, far greater than doses used in dietary supplements, which are typically <0.5 mg/kg/day. Absence of mutagenicity was confirmed in the same study, using the Ames test. In 2011, the GRAS ('Generally Regarded As Safe') status of MZ was acknowledged by the FDA in a reply to a proposal from a US company on the status of MZ (plus L and Z). Finally, a recent safety evaluation of MZ by Xu *et al* concluded that MZ has no acute toxicity and no genotoxicity and the use of MZ is safe at dose of 300 mg/kg body weight per day in rats from a 90 day feeding study. The authors then applied a 100 fold safety factor, and reported an ADI (acceptable daily intake) of 3 mg/kg body weight per day for MZ.⁴³

Conclusion

The conviction that retinal MZ is derived wholly or solely from retinal L is unsafe. Foodstuffs that are likely sources of the macular carotenoids should be

investigated for the presence of L, Z, and MZ using the most modern techniques available to separation science, and any meaningful comment on the bioconversion of these carotenoids within retinal tissue can only be based on the results of supplementation studies that include an intervention arm for each of the three macular carotenoids.

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Sir,
Comment on: What is meso-zeaxanthin, and where does it come from?

A recent article in this journal by Nolan *et al*¹ provides a review of biochemical processes thought to be involved in the deposition of meso-zeaxanthin (MZ) in the human retina. The main purpose of the review was to bring into question current understanding of the source of retinal MZ and that it is derived solely from lutein (L). The paper evaluates publications on the dietary sources of MZ, L, and zeaxanthin (Z)^{2,3} and on two supplementation studies with L and Z in non-human primates⁴ and Japanese quail.⁵ These two latter studies concluded that MZ in the retina is derived solely from L. The review challenges this conclusion, but contains critical errors that should be considered, as detailed below.

Quantitation of xanthophylls using reverse- and normal-phase HPLC

Nolan *et al*¹ argue that the two-step HPLC method used for MZ quantitation by Johnson *et al*⁴ is limited because of the labor involved in the manual collection of the total Z + MZ fraction in the first step. The authors suggest that this process is prone to human error, in that only a portion of the Z + MZ fraction would be collected, and that this fraction typically is contaminated with L carryover. We have found that contamination of the Z + MZ fraction by L, if it occurred, is not a problem. In this chromatographic system, the presence of L does not interfere with Z determination. Baseline separation between L and Z is illustrated in Figure 1B of Johnson *et al*.⁴ Furthermore, precise collection of peaks in the first step of this analysis is not difficult. It is a matter of collecting the eluent before the Z + MZ peak appears on the chromatographic monitor and continuing until after the peak of interest has reached baseline. The fact that L, MZ and Z appear on the subsequent normal phase, chiral column chromatogram verifies that the desired peaks were collected, and this was also confirmed by absorption spectra. However, it is important to note that quantitation is performed on the reverse-phase system (from which peaks are collected) and therefore, precision or total peak collection would not be an issue, and it is not imperative that the entire peak be collected. The relative ratio of MZ to Z is determined from the normal phase, chiral column chromatographic data. Applying that ratio to the quantitative data (obtained from the first-step reverse-phase HPLC results) allows for an estimation of MZ. A more important point to be made in reference to the Nolan *et al*¹ review is that the qualitative data of the Johnson *et al*⁴ paper is of more significant interest than quantitative data. That is, the key question is not how much MZ comes from L but rather whether MZ comes solely from L or not.

Nolan *et al*¹ have concerns about an unknown peak that was found to co-elute with the Z fractions of retinal samples in the report by Johnson *et al*⁴ which they believed could affect their conclusions. As stated in the article by Johnson *et al*⁴ the spectrum of this unknown peak indicated that it is not a carotenoid. This was confirmed by the fact that the peak also appeared in the

reverse-phase HPLC of retinal samples from the carotenoid-free monkeys. Furthermore, the peak did not appear in the normal-phase HPLC system of the carotenoid-free monkey samples or outside the macular area of the L-fed or Z-fed monkeys.⁴

The role of saponification in the quantitation of xanthophylls in food and supplements

Nolan *et al*¹ further question the absence of MZ in foods. They highlight a substantial discrepancy between the extraction methods used in a recent paper by Rasmussen *et al*³, which found no MZ in a variety of seafood, vs those used by Maoka *et al*² that reported MZ in multiple seafood species. The authors state that Rasmussen *et al*³ did not saponify the foods before analysis and that this is a required step in the process of carotenoid extraction, as it frees esterified carotenoids in the food sample that otherwise would not be observed during analysis, thereby resulting in an underestimation of MZ. This claim is not correct. If saponification was not performed, carotenoid esters still would be detected using the method employed by Rasmussen *et al*,³ as indicated by Chung *et al*.⁶ The esters elute after the free L and Z and during the period of detection. In the paper by Maoka *et al*², the significance of the tested foods as dietary sources of MZ cannot be determined because they did not determine the absolute amount of MZ. The reason for the discrepancy between these two papers remains unknown. However, the methods used by Maoka *et al*² are also problematic, because they performed a chemical derivatization to generate analytes that could be separated by normal-phase HPLC. This step could introduce artifacts.

To independently examine the possible presence of MZ in fish, analyses of salmon and trout fillet and skin were conducted in the laboratories of DSM Nutritional Products (Joseph Schierle, personal communication). In brief, the tissues were extracted with acetone or subjected to a short, hot saponification (15 min at 80 °C) in ethanolic potassium hydroxide followed by extraction with diethyl ether. The acetone and ether extracts were evaporated, reconstituted in n-hexane/acetone, and then directly chromatographed with both a chiral HPLC (Chiralpak) and a non-chiral normal-phase HPLC. In general, the total Z (sum of three stereoisomers) concentrations were found to be very low, and MZ was found only in salmon skin. In the fillet, total Z concentrations were ~0.1 p.p.m. (salmon) and 0.3 p.p.m. (trout) and >99% was present as 3R,3'R-Z. Furthermore, in the fillet, Z was present in the free form, whereas in the skin, Z was mainly esterified, with concentrations of ~0.15 p.p.m. (salmon) and 1.7 p.p.m. (trout). After saponification, it was found that the Z in trout skin was >99% 3R, 3'R-Z. However, in salmon skin all three stereoisomers were present with ~15% MZ, 16% 3S,3'S-Z and 69% 3R, 3'R-Z. The diode array detector spectra of all three peaks matched clearly with all-trans Z. In the skin samples, L was present at levels lower than Z (~17% and 24% of total Z present for salmon and trout, respectively). Thus, while MZ was detected in salmon skin only, the amount (0.02 p.p.m.) in this rarely consumed food cannot be considered a significant dietary source of macular pigments.

Meso-zeaxanthin in lutein supplements

Nolan *et al*¹ state that they have detected MZ in commercially available L supplements and that this may have implications for the current understanding of the origins of MZ, as it has been proposed that retinal MZ is derived solely from retinal L. However, this does not address the point in question: 'What is the source of retinal MZ in the normal diet, and does it come solely from dietary L?' One would expect MZ to appear in the macula if a MZ-containing supplement is taken. However, the conclusion that dietary L is the usual source of retinal MZ is confirmed by the finding of Johnson *et al*⁴ that MZ appeared only in the macula of carotenoid-free monkeys fed pure L (as determined by two independent laboratories) and not in carotenoid-free monkeys fed pure Z.

Nolan *et al*¹ suggest that the L supplement used in the Johnson *et al*⁴ study in carotenoid-free monkeys contained MZ, and that this contamination could explain the results. They present this possibility based on their work that identified MZ in Ultra Lutein, which contains DSM-sourced L. This contention is not correct, because the L source used by Johnson *et al*⁴ was not Ultra Lutein, and the paper specifically states that the L supplement had no detectable Z by analytical HPLC. If no Z was present, there could be no MZ present because Z and MZ co-elute on non-chiral HPLC systems and are easily separable by many HPLC methods. Even so, if in fact there were a small amount of MZ present in the supplemented L, the bioavailability would have had to have been extraordinary high (many orders of magnitude higher than L) to explain the substantial amounts present in the central 4-mm retinal sample, which comprised approximately half of the total xanthophyll content. Studies in humans in fact suggest that MZ has a lower bioavailability than L.⁷

Additional evidence supporting lutein as the precursor of meso-zeaxanthin

Lastly, Nolan *et al*¹ state that the hypothesis that retinal MZ is wholly and solely the result of bio-conversion of retinal L is inconsistent with the findings of Bhosale *et al*⁵ who measured deuterated (D) L, D-Z and D-MZ in the retina of quail following supplementation with either D-L, D-Z or regular diet (control group).⁵ Following euthanization, D-L and D-MZ were identified only in animals supplemented with D-L, whereas D-Z was the only isotopically labeled macular carotenoid identified in animals supplemented with D-Z. Nolan *et al*¹ point out that there was a marked discrepancy between the proportions of total retinal L and total retinal MZ that were deuterated (83% *vs* 42%, respectively), suggesting that retinal MZ is not derived exclusively from retinal L. It should be noted that this quail study was designed to demonstrate which compounds are precursors for the various carotenoids found in the retina and other tissues. As the birds were not on a completely carotenoid-free diet before supplementation with the labeled material, the less than 100% labeling is not unexpected. Because the rate of depletion from the retina for endogenous L *vs* Z is not known, not much emphasis can be placed on the difference in deuteration levels because some dilution of the labeling percentage would be anticipated

due to preexisting unlabeled retinal carotenoids, as their half-lives in the tissues are thought to be very long. These studies in quail definitively show that L and not Z is the precursor for MZ and are entirely consistent with the monkey results of Johnson *et al*.⁴

Conclusions

The authors' implication that there may be other dietary sources of natural MZ requires supportive data. The presence of MZ in natural food products needs to be convincingly demonstrated. It is true that there is synthetic MZ in the modern food supply due to Mexican eggs from chickens fed MZ and its presence in small amounts in supplements, but there is no evidence, with the exception of the paper by Maoka *et al*² that fruits, vegetables, or other normally consumed human foods contain MZ. However, the significance of the latter work may be questioned given the lack of quantitation and the inability to replicate its findings. Moreover, MZ is not detectable in human serum or non-ocular tissues of individuals not supplemented with this xanthophyll,^{8,9} which supports the absence of MZ in the natural food supply.

Conflict of interest

The authors declare no conflict of interest.

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Sir,
Response to Bernstein *et al*

We welcome the letter by Bernstein *et al*¹ in response to our publication 'What is meso-zeaxanthin, and where does it come from?' in *Eye* 2013.² In their letter, Bernstein and colleagues argue that our review article contains '*several critical errors that need to be considered.*'

Bernstein and colleagues endeavour to make their points under the following headings:

1. Quantitation of xanthophylls using reverse- and normal-phase HPLC.
2. The role of saponification in the quantitation of xanthophylls in food and supplements.
3. Meso-zeaxanthin in lutein supplements.
4. Additional evidence supporting lutein as the precursor of meso-zeaxanthin.

In our letter below, we reply directly to these points in normal font. Statements made by Bernstein and colleagues are presented in bold font for clarity.

1. Quantitation of xanthophylls using reverse- and normal-phase HPLC.

'Nolan *et al* argue that the two-step HPLC method used for MZ quantitation by Johnson *et al* is limited because of the labor involved in the manual collection of the total Z + MZ fraction in the first step. The authors suggest that this process is prone to human error, that only a portion of the Z + MZ fraction would be collected, and that this fraction typically is contaminated with L carryover.'

We thank Bernstein *et al* for summarising the two-step method in their correspondence, commonly used for quantifying MZ. We are very familiar with this method, as we have used it in several of our recently published studies.^{3–6}

In our review article, we point out the limitations of the standard 'two-step method' commonly used by many laboratories to quantify MZ. These limitations include the following: its labour intensive nature due to manual collection; operator dependency and potential for human error; and a very long sample run time, rendering it difficult to perform bulk analysis (eg, for clinical trials). Our concerns with respect to the traditional 'two-step method' remain, and we believe that it is important to recognise these limitations when discussing published methodology and findings from papers, and that is why we included these points in our review.

Bernstein *et al* premise their defence of the methodology of carotenoid quantification in the paper by Johnson *et al*⁷ on the basis that:

'The fact that L, MZ and Z appear on the subsequent normal-phase, chiral column chromatogram verifies that the desired peaks were collected, and this was also confirmed by absorption spectra.'

Bernstein *et al* attempt to address our concerns with respect to the unknown peak that was found to co-elute with the Z fractions of retinal samples in the report by Johnson *et al*⁷ by stating that '*... the peak also appeared in*

the reverse phase HPLC of retinal samples from the carotenoid-free monkeys.'

We agree that identifying the peaks and confirming their presence by assessing their absorbance spectra are important. However, it is clear from the Johnson *et al*⁷ paper that the already challenging method used to analyse MZ was made more difficult by the presence of the unknown peak. The authors did, however, attempt to address this issue using a customised equation that incorporated L and Z ratios to adjust for the presence of the unknown peak.

Indeed, Johnson *et al* concede to this limitation in their paper, as follows:

'This fact introduces an inherent limitation in the precision of our estimates, but as explained later, it affects only the estimates of RRZ in the Z-fed group. Bearing in mind the limitations of our estimates, we found that all samples from the Z-fed animals had higher concentrations of RRZ than did the control subjects, and the differences between Z-fed and control animals for the 8-mm and the peripheral samples were statistically significant (Table 4).'

2. The role of saponification in the quantitation of xanthophylls in food and supplements.

In our review, we point out that in the study by Rasmussen *et al*⁸ (which concluded that MZ, L, or Z were not present in fish or seafoods) that the investigators had failed to saponify their samples, and therefore would be unable to detect these carotenoids (if present). Indeed, data from our laboratory clearly show the need to saponify in order to detect MZ, Z, or L in fatty samples (eg, fish) containing esterified MZ, Z, or L (see below).

With respect to the role of saponification for the purposes of carotenoid quantitation, Bernstein *et al* contend that:

'If saponification was not performed, carotenoid esters still would be detected using the method employed by Rasmussen *et al*, as indicated by Chung *et al*. The esters elute after the free L and Z and during the period of detection.'

Such a contention may indeed be the case for reverse-phase conditions, where, typically, the nonpolar compounds, such as esterified carotenoids, elute after the more polar-free form carotenoids. Esterified carotenoids typically cluster in chromatographs, and as multiple esters can arise from the esterification of Z and/or its isomers *in vivo*, it is impossible (unless specific standards are used) to accurately distinguish a zeaxanthin ester from a MZ ester under either reverse- or normal-phase conditions. In addition, and having reviewed the paper by Chung *et al* referred to by Bernstein *et al*, it is worth noting that the subject matter of the cited paper, in fact, is limited to lutein and makes no reference to zeaxanthin or its isomers in either free or esterified form.

Next, Bernstein *et al* criticise the methodology of Maoka *et al*⁹ on the basis that:

'However, the methods used by Maoka *et al* are also problematic, because they performed a chemical derivatization to generate analytes that could be

separated by normal phase HPLC. This step could introduce artifacts.'

It is noteworthy that Bernstein *et al* cite a personal communication relating to research performed in the laboratories of DSM Nutritional Products in order to '...independently examine the possible presence of MZ in fish'. Indeed, and even under such circumstances and where derivatisation was not employed, the DSM scientists identified the presence of MZ in salmon skin, thereby confirming the occurrence of this carotenoid in the human food chain. Their finding is, indeed, consistent with data from our laboratory (see Figure 1, showing the presence of a peak with the same spectrophotometric characteristics and retention time of MZ in salmon skin; note, we have also identified MZ in other marine species, but we present just one example here for the purpose of this reply). Of note, our findings are consistent with all the published literature reporting on MZ in fish (see Schiedt *et al*,¹⁰ Maoka *et al*,⁹ and Katsuyama *et al*¹¹) with the sole exception of the recent paper by Rasmussen *et al*,⁸ which did not detect MZ in such marine species. However, as explained above and in our Review,² we believe that the failure to saponify the fish and seafood samples tested precluded the identification of L, Z, and MZ in the foods tested.

3. Meso-zeaxanthin in lutein supplements.

Nolan *et al* suggest that the L supplement used in the Johnson *et al* study in carotenoid-free monkeys contained MZ, and that this contamination could explain the results. They present this possibility based on their work that identified MZ in Ultra Lutein, which contains DSM-sourced L. This contention is not correct, because the L source used by Johnson *et al* was not Ultra Lutein, and the Johnson *et al* paper specifically states that the L supplement had no detectable Z by analytical HPLC.

We did not say the study conducted by Johnson *et al* used Ultra Lutein; rather, we are simply pointing out that the L standards and supplements that we have tested, which were sourced from Kemin/DSM, typically contain MZ.

Indeed, we have now tested many DSM/Kemin lutein-containing supplements, and the majority of the samples we analysed contain undeclared MZ. Moreover, we have also tested the L standards kindly provided by DSM, which are used in our laboratory for calibration,

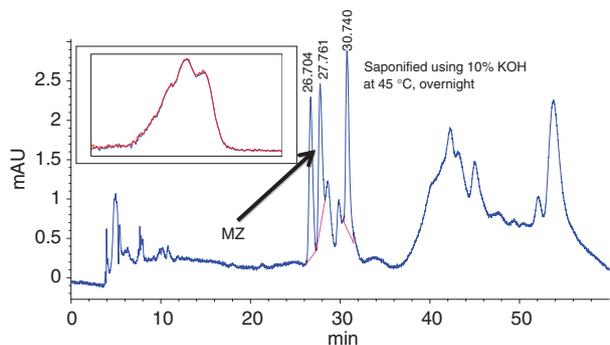


Figure 1 Salmon skin sample.

and these standards also contain undeclared MZ.

Importantly, our finding of undeclared MZ in a supplement formulation (Ultra Lutein) had implications for one of our recently published supplementation trials, and hence why we felt the need to discuss this discovery in our review.²

Indeed, these findings that MZ is present in some brands of commercially available L supplements (and not declared) remain uncontested by the supply company, and remain a concern with respect to the conclusions of Johnson *et al*,⁷ as it is possible that the 'pure L' feed did in fact contain some MZ.

In addition, it is noteworthy that the chromatograph of 'pure L' was not presented in the publication by Johnson *et al*. Further, we have invited DSM to provide this chromatography, but this request has not yet been met.

4. Additional evidence supporting lutein as the precursor of meso-zeaxanthin.

To date, the hypothesis that retinal MZ is derived from retinal L is supported by only two studies, one in Rhesus monkeys and the other in quail. Furthermore, these studies were not designed to investigate, even in those nonhuman species, whether retinal MZ was derived 'wholly and solely' from retinal L, for the reasons outlined in our review.²

In summary, we have challenged the received wisdom that retinal MZ in humans is derived wholly and solely from retinal L. We believe that Bernstein *et al* would concur that there is a paucity of data on the origins of MZ in human retina, and invite these distinguished commentators and colleagues to join with us in our concluding plea of the Abstract of our review paper, namely: 'Certainly, the narrative that retinal MZ is derived wholly and solely from retinal L needs to be revisited.'

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