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 primates[4] 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 was ~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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PS Bernstein[1], EJ Johnson[2], M Neuringer[3], W Schalch[4] and J Schierle[4]

[1]Ophthalmology and Visual Sciences, Moran Eye Center, University of Utah School of Medicine, Salt Lake City, UT, USA
[2]Carotenoids and Nutrition Laboratory, Jean Mayer USDA Human Nutrition Research Center on Aging, Tufts University, Boston, MA, USA
[3]Ophthalmology and Clinical Nutrition, Oregon Health & Science University, Portland, OR, USA
[4]DSM Nutritional Products, Basel, Switzerland
E-mail: elizabeth.johnson@tufts.edu

Eye (2014) **28,** 240–242; doi:10.1038/eye.2013.257; published online 6 December 2013

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,