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

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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 Katsuyma *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.

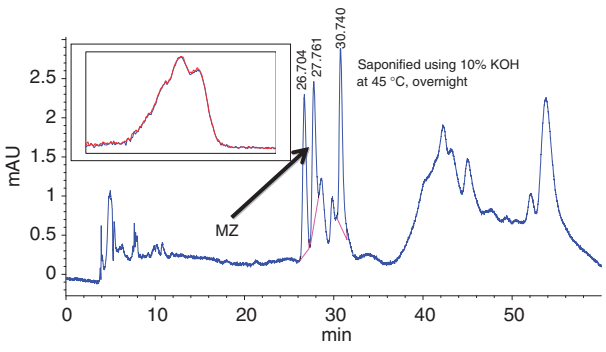

Figure 1 Salmon skin sample.

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, 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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Sir,
Intravitreal foreign body following intravitreal anti-VEGF injection: a case report

Since the advent of anti-VEGF, it has been widely used for the management of macular edema, especially diabetic macular edema. Bevacizumab being used off-label for that purpose. Rare complications following intravitreal injections include: endophthalmitis, uveitis, retinal tear and retinal detachment.[1]

Case report

We report a case of a 50-year-old diabetic male patient with diabetic macular edema. He received intravitreal injections of Bevacizumab for five times: three to his right eye and two to the left eye.

Two months after the last intravitreal injection to his right eye, the patient complained of seeing a floater in that eye. Past ophthalmic history included only intravitreal injections and central laser treatment. No intraocular surgeries were performed for him, and there was no history of ocular trauma.

Anterior segment showed no signs of inflammation. Posterior segment exam showed bilateral, severe, nonproliferative diabetic retinopathy. In the right eye, a fine cotton fiber with a length of less than one optic disc diameter was suspended in the posterior vitreous towards the temporal side. No evidence of inflammation in the posterior segment was seen.

Colored fundus photographs showed the short fiber floating in the vitreous, Figure 1. Colored fundus video was done for documentation.

During a 5-month follow-up period, no signs of inflammation were noticed.

Comment

Previous reports described the appearance of silicone oil droplets in the vitreous which were asymptomatic and

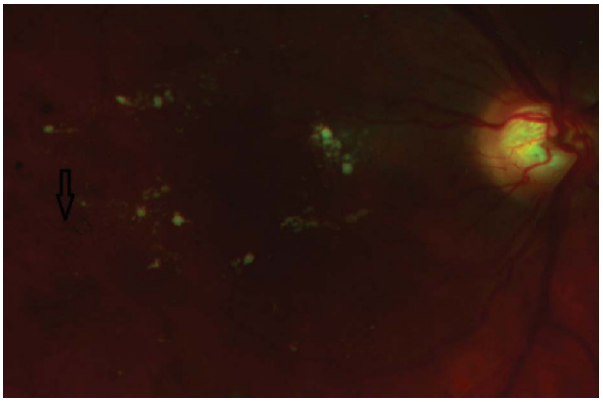

Figure 1 Intravitreal foreign body; a fiber suspended in the posterior vitreous (arrow).