

RESEARCH ARTICLE

Lutein, zeaxanthin and *meso*-zeaxanthin content of eggs laid by hens supplemented with free and esterified xanthophylls

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(Received 5 June 2015 – Final revision received 1 October 2015 – Accepted 27 October 2015)

Journal of Nutritional Science (2016), vol. 5, e1, page 1 of 10

doi:10.1017/jns.2015.35

Abstract

The xanthophyll carotenoids lutein (L), zeaxanthin (Z) and *meso*-zeaxanthin (MZ) are found at the macula, the central part of the retina, where they are referred to as macular pigment (MP). MP is studied in human subjects because of its proven role in enhancing visual function and its putative role in protecting against age-related macular degeneration. These benefits are probably due to the antioxidant and short-wavelength filtering properties of MP. It is known that eggs are a dietary source of L and Z. This experiment was designed to measure the egg yolk carotenoid response to hen supplementation with L, Z and MZ. A total of forty hens were used in the trial and were divided into eight groups of five hens. Each group was supplemented (with about 140 mg active xanthophylls/kg feed) with one of the following oil-based carotenoid formulations for 6 weeks: unesterified L (group 1); L diacetate (group 2); unesterified Z (group 3); Z diacetate (group 4); unesterified MZ (group 5); MZ diacetate (group 6); L–MZ (1:1) diacetate mixture (group 7); L–MZ diacetate (1:3) mixture (group 8). Yolk carotenoid content was analysed weekly (in four randomly selected eggs) by HPLC. We found that hens supplemented with Z diacetate and MZ diacetate produced eggs with significantly greater carotenoid concentrations than their free form counterparts. This finding potentially represents the development of a novel food, suitable to increase MP and its constituent carotenoids in serum.

Key words: Lutein: Zeaxanthin: *Meso*-zeaxanthin: Lutein diacetate: *Meso*-zeaxanthin diacetate: Zeaxanthin diacetate: Egg fortification: Hen supplementation

The xanthophyll carotenoids lutein (L), zeaxanthin (Z) and *meso*-zeaxanthin (MZ) are found at the macula⁽¹⁾, the central part of the retina responsible for fine detail and central vision⁽²⁾. At this specialised location, they are referred to as macular pigment (MP) and, via their short-wavelength (blue) light filtration⁽³⁾ and antioxidant properties⁽⁴⁾, protect against age-related macular degeneration (AMD)⁽⁵⁾. In addition, MP augmentation in humans has been shown to improve visual

function in the AMD (diseased) retina^(6–8) and in the non-diseased (healthy) retina⁽⁹⁾. Also, studies have shown that L and Z are found in the human brain^(10,11), and recent reports suggest a role for these xanthophylls in supporting brain health and cognitive function^(11–13). Of note, the majority of studies to date have demonstrated MP augmentation and consequential visual benefits using over-the-counter carotenoid supplement preparations. Of interest, however, nutrient-enriched

Abbreviations: CisZ, 13-*cis*-zeaxanthin; L, lutein; MP, macular pigment; MTBE, methyl *tert*-butyl ether; MZ, *meso*-zeaxanthin; Z, zeaxanthin.

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(functional) foods may offer an alternative to food supplements as can be seen, for example, with fatty acids (e.g. *n*-3 and *n*-6), which are now available from enriched eggs and milk^(14,15).

L and Z are present in a typically varied diet⁽¹⁶⁾, whereas MZ is only found in trace amounts in marine-sourced foods, such as salmon, trout and sardines^(17,18). Despite the differing amounts of L, Z and MZ found in foods, it is interesting to note that the ratio of these xanthophylls in the human retina is 1:1:1 (MZ:Z:L), suggesting a unique role for each of these carotenoids at this target tissue. Foods such as kale, spinach and maize contain high amounts of L and Z, rendering such foods important sources of these carotenoids. However, the complex cellular structure of these higher plants impedes the release of the chromoplast-bound carotenoids⁽¹⁹⁾. It is for this reason that the bioaccessibility and bioavailability of these compounds need to be enhanced in order to increase their concentrations in serum. This can be achieved by optimising the mode of food preparation; for example, chopping, maceration, cooking of the plant tissue in question, and incorporation of oil when eaten^(20,21).

Of interest, although hen eggs contain lesser concentrations of L and Z than some leafy greens (e.g. spinach or kale)⁽¹⁶⁾, the high lipid content of the egg facilitates gastrointestinal digestion and absorption, and enhances the bioavailability of the carotenoids present in the yolk, making eggs a good source of these nutrients⁽²²⁾. As a consequence, greater serum carotenoid responses have been observed following supplementation with egg yolk when compared with dietary fortification with spinach/kale extracts and even when compared with oil-based supplement preparations in human clinical trials⁽²³⁾. Also, in that same study by Chung *et al.*⁽²³⁾, it was reported that L bioavailability was comparable between free L and L esters.

Hen eggs are consumed as part of a typical healthy diet and are produced on an industrial scale to meet the high demand of egg and egg-based products on the market. A golden-yellow egg yolk is sought-after⁽²⁴⁾, and the carotenoids L, Z and MZ are already added to hen feed (e.g. Industrial Orgánica) in order to enhance the cosmetic appearance and commercial value of the egg yolk, i.e. the increased carotenoid pigment gives rise to a richer yolk colouration. Also, it is possible that carotenoid-enriched eggs will offer advantages over standard eggs in terms of increasing MP levels, with consequential benefits for vision and cognitive function. It is known that, in general, supplementation with carotenoids results in increases in serum and retinal concentrations of the carotenoids. This has been demonstrated with carotenoid-rich foods, such as eggs^(25,26), and with carotenoid supplements containing L, Z and MZ^(6,9,27,28).

However, it is not known if the response of hens to esterified carotenoids differs with respect to their egg yolk carotenoid concentration, when compared with carotenoids in their free form. In 2002, Bowen *et al.* reported higher bioavailability in humans from a L ester formulation, when compared with the free form (61% more bioavailable; $P < 0.05$)⁽²⁹⁾. In 2009, Wu *et al.* reported that L bioavailability, measured in hen plasma, from free L and esterified L

supplements was comparable⁽³⁰⁾. However, their experiment did not analyse the impact of supplementation in the eggs of the hens supplemented with the esterified and free L supplements. Of note, the present study is the first study to compare L, Z and MZ response in eggs of hens supplemented with free and esterified L, Z and MZ. On one hand, the free form of the carotenoids may offer advantages over the esterified carotenoids as they are readily available for absorption from the gut into the bloodstream, whereas esterified carotenoids need to undergo enzyme-catalysed hydrolysis, and there is little known about the digestion, absorption and metabolism of the esterified carotenoids. On the other hand, the esterified carotenoids may offer advantages over the free-form carotenoids because esterified carotenoids are known to be more stable than the free-form carotenoids, and are not degraded, to the same degree, by factors such as light and heat. Overall, knowledge in this area is limited and the present study is designed to address some of these important questions.

The present trial, known as the Egg Xanthophyll Intervention Trial (EXIT), was designed to measure egg yolk carotenoid response to hen supplementation with L, Z, and MZ in their free and esterified forms, including testing mixtures of esterified L and MZ on egg yolk carotenoid response.

Methods

Housing and feed conditions

The experimental methods implemented in this study conformed to Directive 2010/63/EU of the European Parliament and Council (22 September 2010) on the protection of animals used for scientific purposes. This study was reviewed and approved by the Ethics Committee of the Waterford Institute of Technology, Waterford, Republic of Ireland.

This hen supplementation trial was housed in a purpose-built barn (28 × 12 feet; 8.5 × 3.7 m) on a farm, which was quality assured by the Food Safety Authority of Ireland (Bord Bia), and which complied with all health standards prescribed by Bord Bia, including *Salmonella* testing. The hens were housed in large indoor pens to allow free movement. Inside the barn there were eight pens, which measured 8 feet deep × 4 feet wide (2.4 m deep × 1.2 m wide) with can feeders and overhead nipple drinkers (*ad libitum*). Plastic slats were used for manure, in that the hens stood on them and the manure fell down between the slats (there was no litter material used to avoid contamination from the manure). The temperature of the bird house was approximately 18°C. A light program of 15 h light per d was used in this trial, consistent with the standard practice of the hen farm.

The hens were of the Goldline variety, known to be excellent egg layers, and were all approximately 40 weeks of age at the start of the trial. All the hens were purchased from a local farmer, and were vetted prior to the commencement of the trial to ensure that the hens used in the trial were healthy. Their diet consisted of a standard commercially available 'crumbled grain' feed (High Performance Layers Mash,



Southern Mills). This crumbled grain was selected over a pelleted feed due to its suitability for mixing with the oil-based carotenoid supplement. A 'carotenoid-free' feed was initially considered, but this concept was discarded in order to keep the trial conditions similar to those of typical egg production. The hens had an adaptation period of 1 week in the barn, where they were fed a standard diet containing the following ingredients: wheat meal; soya (bean) meal; maize; calcium carbonate (lime grit); distillers dried grains; barley; rapeseed meal; sunflower seed meal; mono-dicalcium phosphate; sodium chloride; animal fat; sodium bicarbonate. The composition of the nutrients was as follows: crude protein 16.0%; crude oils and fats 2.7%; Ca 3.80%; Na 0.17%; crude fibre 4.2%; crude ash 12.6%; lysine 0.68%; P 0.56%; methionine 0.32%.

Hen intervention groups and feed supplements

The forty hens involved in the trial were divided into eight groups of five hens and each group was supplemented with one of the following oil-based carotenoid formulations: unesterified L (group 1); L diacetate (group 2); unesterified Z (group 3); Z diacetate (group 4); unesterified MZ (group 5); MZ diacetate (group 6); L–MZ (1:1) diacetate mixture (group 7); L–MZ diacetate (1:3) mixture (group 8). The free carotenoids and their diacetates are presented in Fig. 1. The two intervention 'mixture' groups each comprised a mixture of L and MZ diacetates, as follows: group 7 in a L:MZ ratio of 1:1 and group 8 in a L:MZ ratio of 1:3, prepared in-house. The trial supplements were supplied by Industrial Orgánica and generally recognized as safe-approved by the Food and Drug Administration (lutein and zeaxanthin (3R,3'R) mixture: GRN 00029; lutein diacetate: GRN 000432; *meso*-zeaxanthin: GRN 000481). The supplements consisted of the carotenoid of interest (e.g. Z is the carotenoid of interest in groups 3 and 4), henceforth referred to as the 'target carotenoid', suspended in maize oil, in a declared concentration of approximately 20 g/kg. Vitamin E (DL- α -tocopheryl acetate; 5 g/kg) was also included in the formulations as a preservative due to its strong antioxidant activity. These supplements were analysed in our laboratory after hydrolysis in order to quantify the specific L, Z and MZ content in their free form, to determine the amount of feed concentrate to add to the feed mix and produce 140 mg/kg. The resulting concentrations and the declared label content of the supplements are presented in Table 1.

Hen feed mixing

The carotenoid concentration of each of the six supplements was determined and was controlled in order to achieve a uniform dosage of 140 mg/kg (mg of active carotenoid per kg of hen feed) across all intervention groups. Since the supplement was added to the feed via 100 ml syringes, and given that the supplements had differing densities, the required quantity of the supplements to be added directly to the feed was converted to volume (ml) using the measured densities provided on the supplement labels and the measured concentrations

of xanthophylls as obtained by HPLC. In the case of the mixture groups, the overall dosage was 140 mg/kg (i.e. 1:1 mixture contained 70 mg/kg of L and 70 mg/kg of MZ). Since some L was detected in the MZ supplement and vice versa, we included the amounts in our calculations.

The hen feed for all eight groups was prepared on a Wednesday, fortnightly. Each 14 kg batch of feed mixed provided 2 weeks' rations per group. The feed was mixed according to a predetermined mix-and-wash protocol to prevent cross-contamination between intervention groups. A planetary mixer (Gastrorag B40A; Commercial Refrigeration) set at medium speed and a 100 ml syringe was used to incorporate the supplement. The feed and supplement were mixed for 10 min to ensure that a homogeneous mixture was produced, which was indicated by the uniform golden colour of the feed. The feed was then divided into 1 kg portions (a daily portion per group of five hens), vacuum-packed (CC771 Buffalo Vacuum packer) and stored at -20°C . A daily portion was provided to the hens in the morning and the feed remnants discarded the next morning to help reduce degradation of the carotenoids in the feed while it was exposed to air.

Sample collection and storage

Egg samples were collected at baseline (before the introduction of experimental diet) and on a weekly basis for a period of 6 weeks thereafter. A sample of four eggs was taken from each of the eight intervention groups at each visit. These eggs were taken from the batch of eggs laid on the day of collection. These egg samples were placed in designated 'group' trays and transported to the laboratory facility to undergo the yolk sample preparation step (described below) that day.

Analytical methods

The following analytical methodologies took place under amber light conditions (eW Blast Powercore BCP473; Philips Electronics Ireland Ltd), and in a temperature-controlled environment (15 – 19°C) to minimise carotenoid degradation.

Hen feed supplement analysis

The six hen feed supplements used in the trial were sealed in individual 5 kg containers upon delivery to the laboratory. These containers were placed in a 40°C water bath for 1 h (in accordance with label instruction), then subjected to mixing by inversion prior to breaking the seal. Quantities of 50 mg were taken in triplicate from each supplement container and each sample was dissolved in 50 ml of acetone. A volume of 1 ml of the resulting solution was removed to a 25 ml volumetric flask and also made up in acetone. Then 0.5 ml of this working solution was transferred to a clear 1.5 ml Eppendorf tube, to which was added 0.1 ml of internal standard (echinenone, 0.4 mg/10 ml ethanol) and 0.5 ml of aqueous KOH (25 g/100 ml water). The samples were allowed to saponify for 1 h at 45°C in a shaking incubator (Stuart

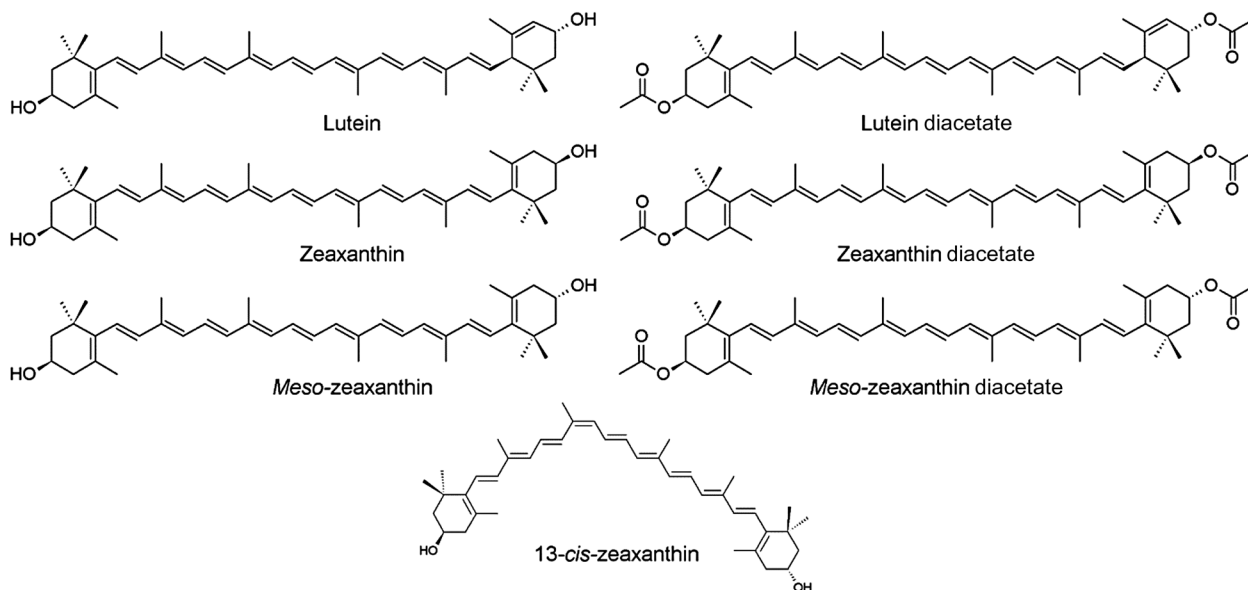


Fig. 1. Chemical structures of the free-form carotenoids and their diacetates.

Orbital Incubator, SI500; Carl Stuart Ltd) and then allowed to cool to room temperature prior to extraction. A quantity of 0.5 ml of hexane containing 0.1 % butylated hydroxytoluene (BHT; 100 mg/l) was added as the extraction solvent to each sample, and the mixture was vortexed (VortexGenie; Carl Stuart Ltd) for 2 min and centrifuged with an AccuSpin Micro 17 (Fisher Scientific) at 400 g for 5 min. Then 0.5 ml of the yellow upper layer was transferred to an amber 1.5 ml Eppendorf tube and the liquid extraction was repeated. The two extracts were combined and dried using a vacuum solvent concentrator (MiVac, GeneVac; Mason Technologies). The samples were immediately reconstituted in 0.2 ml (0.1 ml injection volume) of mobile phase (see assay 1) for HPLC analysis.

Egg yolk preparation

Egg yolks were individually weighed and also colour graded using the DSM Colour fan. The yolks were thoroughly

mixed with PBS (0.01 M, pH 7.4) and made up to 50 ml in a volumetric flask. Then 3 ml of each suspension was transferred to two clear 1.5 ml Eppendorf tubes and stored at -80°C until analysis.

Yolk carotenoid extraction

Four eggs from each group were analysed for carotenoid content, and each individual egg yolk sample was analysed without replication. The egg yolk suspension (0.1 ml) was mixed with 0.15 ml aqueous KOH (25 g/100 ml water), 0.15 ml absolute ethanol (containing BHT, 500 mg/l) and 0.1 ml echinenone (internal standard, 0.4 mg/10 ml ethanol) in a clear 1.5 ml Eppendorf tube and incubated at 45°C for 45 min in a shaking incubator (Stuart Orbital Incubator, SI500; Carl Stuart Ltd). The samples were then cooled and vortexed vigorously (VortexGenie; Carl Stuart Ltd) with 0.5 ml of a 1:1 mixture of methyl *tert*-butyl ether (MTBE) and hexane and centrifuged

Table 1. Carotenoid content of each hen feed supplement preparation (Mean values and standard deviations)

Supplement	Density*	Measured carotenoid content†								Declared carotenoid content‡	
		L (g/kg)		Z (g/kg)		MZ (g/kg)		TZ (g/kg)		L (g/kg)	TZ (g/kg)§
		Mean	SD	Mean	SD	Mean	SD	Mean	SD		
MZ (free)	0.92	14.65	1.49	4.98	0.19	36.60	4.58	41.58	4.77	11.59	24.37
Z (free)	0.92	5.68	0.03	42.02	2.47	0.00	0.00	42.02	2.47	3.48	29.65
L (free)	0.91	37.45	0.33	2.75	0.05	0.31	0.05	3.06	0.09	24.77	2.62
MZ diacetate	0.92	2.99	0.46	2.25	0.33	25.81	3.12	28.06	3.46	4.66	25.46
Z diacetate	0.92	1.95	0.28	16.82	0.89	0.00	0.00	16.82	0.89	3.45	23.78
L diacetate	0.95	23.95	1.88	1.28	0.04	2.39	0.36	3.67	0.40	20.54	3.6

L, lutein; Z, zeaxanthin; MZ, *meso*-zeaxanthin; TZ, total zeaxanthin (mixture of MZ and Z).

* Density refers to the density of the liquid supplement at room temperature.

† Measured carotenoid content refers to the carotenoid concentrations obtained by supplement analysis after hydrolysis, performed in-house; samples were analysed in triplicate.

‡ Declared carotenoid content refers to the label-declared carotenoid concentrations given by the supplement supplier; the g/kg unit refers to g active (carotenoid) per kg oil.

§ MZ and Z content was given as a combined (TZ) content on the supplement label.



in an AccuSpin Micro 17 (Fisher Scientific) for 5 min at 400 g to separate the organic and aqueous layers. Then 0.4 ml of the upper organic layer was transferred to an amber 1.5 ml Eppendorf tube and the residue was re-extracted with 0.5 ml of the MTBE–hexane mixture. After centrifuging, 0.4 ml of the upper layer was removed and combined with the first extract, then dried using a vacuum solvent concentrator (MiVac, GeneVac; Mason Technologies). The samples were stored under -80°C conditions until the time of HPLC analysis, which was completed within 1 d of extraction. Samples were reconstituted in 0.2 ml of mobile phase A (see assay 1) and 0.05 ml was used as the injection volume⁽³¹⁾.

HPLC (assay 1)

The Agilent 1260 Infinity HPLC system (Agilent Technologies) was used for assay 1 analysis, and was equipped with the following: binary pump with an adjacent degasser; a diode array detector; temperature-controlled autosampler; temperature-controlled column compartment; and fraction collector.

All extracted samples (both hen feed supplement and yolk samples) were first run on assay 1, a reverse-phase method capable of separating the main carotenoid structures, i.e. xanthophylls and carotenes, as well as *trans*- and *cis*-carotenoid structures (using a method modified from Yeum *et al.*⁽³¹⁾). All-*trans* L and total all-*trans* Z (a mixture of the stereoisomers Z and MZ) were separated and quantified using this technique. A YMC C30 column (250×4.6 mm, $3 \mu\text{m}$) with a 10×4 mm, $3 \mu\text{m}$ YMC Guard Cartridge (guard and column; Apex Scientific) maintained at 15°C was used for this analysis. The gradient method used a 83:15:2 mixture of methanol (MeOH)–MTBE–water as solvent A, and a 8:90:2 mixture of MeOH–MTBE–water as solvent B. The initial isocratic settings were 90 % A and 10 % B for 17 min, followed by a linear increase to 60 % B between 17 and 25 min. The system was then allowed to return to the initial settings over 2 min prior to the next injection. A consistent flow rate of 1 ml/min was used throughout the analyses. L typically eluted at approximately 7 min and total Z at approximately 8.7 min. The total Z peak was automatically recognised and collected in Eppendorf tubes by the system's fraction collector, then dried down using the solvent concentrator (MiVac, GeneVac; Mason Technologies). The collected total Z samples were stored under -80°C until analysis on assay 2.

HPLC (assay 2)

The Agilent 1200 HPLC system was used for assay 2 analysis, and was equipped with the following: quaternary pump with an adjacent degasser; a diode array detector; temperature-controlled autosampler; temperature-controlled column compartment. Assay 2 is a normal-phase chiral method incorporating a Daicel AI-3 CHIRALPAK 250×4.6 mm, $3 \mu\text{m}$ column with a $0.5 \mu\text{m}$ in-line filter used for the chiral separation of Z and MZ. All the collected total Z fractions were analysed for MZ. This gradient method used *n*-hexane as solvent A and iso-propanol as solvent B with a flow rate of

0.8 ml/min. The initial settings were 95:5 hexane–isopropanol, which was isocratic for the first 5 min, then developed a slow linear gradient to 10 % isopropanol over the next 20 min. The system then plateaued at 15 % isopropanol for 5 min, before returning to the initial settings for 4 min in preparation for the next injection. MZ and Z elute at 27.7 and 28.5 min, respectively. The MZ:Z ratio was achieved using their associated peak areas in assay 2, and was applied to the concentration of total Z obtained in assay 1 in order to individually quantify MZ and Z. This is known as the method of proportions technique⁽³²⁾.

Statistical analysis

The statistical packages SPSS (SPSS version 19; IBM version 21; SPSS), were used for data analysis. Between-group differences for yolk carotenoid concentrations, and also yolk weight, were calculated at baseline, and at week 6 using *post hoc* one-way ANOVA. For the within-group analysis of changes over time, repeated-measures ANOVA and paired *t* tests could not be performed due to the hen housing system used, which allowed the hens within a specific group to mingle freely, so that eggs from individual hens in each group could not be identified at the different time points. Linear mixed models, with hen group as 'subject' variable, were also ruled out because, in this study, the treatment groups coincided with the hen groups. Therefore, differences over time (between baseline and week 6) for yolk weight and yolk carotenoids were investigated using independent-samples *t* tests; statistically, these are more stringent tests than paired *t* tests. The 5 % level of significance was used throughout all analyses, without adjustment for multiple comparisons.

Results

Hen feed supplement analysis

The measured *v.* declared L, Z and MZ carotenoid concentration (in g/kg of active carotenoid in maize oil) is given in Table 1. The measured values were used to prepare the dosages given to the hens in order to achieve the uniform 140 mg/kg dosage across all eight intervention groups.

Yolk carotenoid analysis, baseline and week 6

Baseline. The baseline levels of L, Z, MZ and 13-*cis*-zeaxanthin (CisZ), presented as a mean value of four eggs of the same hen group, are given in Table 2. At baseline, there was no significant difference between the eight intervention groups in terms of L ($P = 0.446$), Z ($P = 0.352$), CisZ ($P = 0.075$), total carotenoid ($P = 0.091$) or yolk weight ($P = 0.634$). Yolks in all groups at baseline were between DSM colour grades 12 and 14. MZ was not detected at baseline in any of the yolk samples tested.

Week 6. For each of the supplemented carotenoids L, Z and MZ, we compared week 6 with baseline using independent-samples *t* tests; other approaches (e.g. paired



Table 2. Baseline carotenoid levels and yolk weights for the eight hen intervention groups* (Mean values and standard deviations)

Group (n 4)	L (µg/yolk)		Z (µg/yolk)		CisZ (µg/yolk)		TC (µg/yolk)		YW (g)	
	Mean†	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
1: Free L‡	108.54	11.99	89.46	4.9	20.84	1.71	218.84	18.47	17.5	0.58
2: L diacetate	131.67	40.59	94.68	13.15	23.4	3.67	249.75	56.92	16.5	1.3
3: Free Z	132.61	37.68	91	20.44	21.04	4.26	244.64	60.61	16.67	1.12
4: Z diacetate	108.54	11.07	87.4	16.84	21.23	4.6	217.17	30.72	16.25	0.96
5: Free MZ	117.49	4.29	94.3	6.4	22.38	1.31	234.18	11.57	16.5	0.58
6: MZ diacetate	107.53	1.42	76.8	10.82	18.86	3.41	203.19	15.63	15.75	1.5
7: 1:1 mixture§	128.8	21.32	102.11	27.66	27.79	5.41	258.7	53.84	16.75	1.26
8: 1:3 mixture	108.09	6.11	77.98	8.73	20.2	2.42	206.27	16.35	17	1.83
P¶	0.446		0.352		0.075		0.383		0.656	

L, lutein; Z, zeaxanthin; CisZ, 13-*cis*-zeaxanthin; TC, total carotenoid (sum of L, Z, MZ and CisZ); YW, yolk weight; MZ, *meso*-zeaxanthin.

* No MZ was identified in baseline yolk samples and was therefore not included in the Table.

† Mean refers to the mean carotenoid content of four eggs; each individual egg yolk sample was analysed without replication.

‡ Free refers to the specified carotenoid in its free form.

§ 1:1 mixture refers to the 1:1 mixture of L and MZ diacetates.

|| 1:3 mixture refers to the 1:3 mixture of L and MZ diacetates.

¶ Statistical significance between groups, tested using *post hoc* one-way ANOVA.

tests) were ruled out as explained above (Statistical analysis section). We obtained a significantly positive ($P < 0.001$) time effect in all cases, suggesting strongly that the carotenoid concentrations in all L-, Z- and MZ-supplemented groups increased significantly over the duration of the study, albeit to varying extents. The specific carotenoids of interest and total carotenoid (defined as the sum of L, Z, MZ and CisZ) responses of all eight intervention groups are graphically presented in Fig. 2. The absolute value (in µg/yolk) achieved

by each intervention group is given in Table 3. Of the interventions tested, the Z diacetate resulted in the maximum relative increase in yolk carotenoid concentration, represented by a total carotenoid concentration of 2528 (SD 237) µg/yolk at week 6. Both mixture groups were effective responders, and, of interest, group 7 (the 1:1 mixture of L:MZ diacetate) achieved a L:MZ ratio (1:1) in the yolk samples. This can be seen in the chromatographic separation of a group 7 (1:1 mixture group) yolk extract presented in Fig. 3.

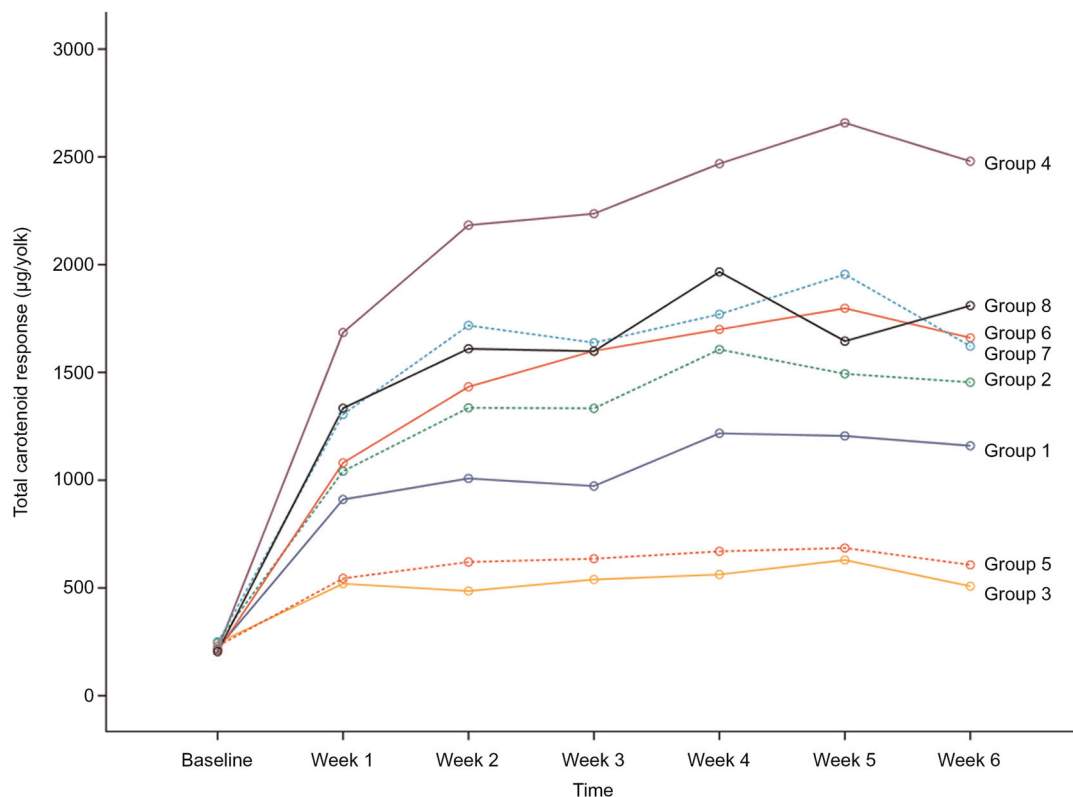


Fig. 2. Graphical presentations of the total carotenoid response in µg/yolk over the 6-week study period. Group 1 = free lutein (L); group 2 = L diacetate; group 3 = free zeaxanthin (Z); group 4 = Z diacetate; group 5 = free *meso*-zeaxanthin (MZ); group 6 = MZ diacetate; group 7 = 1:1 mixture of L diacetate and MZ diacetate; group 8 = 1:3 mixture of L diacetate and MZ diacetate. Total carotenoid response is defined as the sum of L, Z, MZ and 13-*cis*-zeaxanthin responses.



Table 3. Yolk carotenoid response to the eight different interventions, at 6 weeks* (Mean values, standard deviations and percentage increases in carotenoid content between baseline and week 6)

Group (n 4)	L (µg/yolk)			Z (µg/yolk)			MZ (µg/yolk)			CisZ (µg/yolk)			TC (µg/yolk)			YW (g)		
	Mean†	SD	%	Mean	SD	%	Mean	SD	%	Mean	SD	%	Mean	SD	%	Mean	SD	%
1: Free L‡	964.07	131.97	788	153.21	20.94	71	5.5	6.7	34.4	3.45	65	1157.19	157.87	429	18.25	1.26	4	
2: L diacetate	1111.39	234.12	744	206.58	43.56	118	74.79	18.43	61.65	17.99	163	1454.4	311.81	482	17.75	1.26	8	
3: Free Z	113.6	14.26	-14	329.4	24.6	262	-	-	65.08	5.09	209	508.07	29.78	108	16.75	1.5	0	
4: Z diacetate	334.5	37.83	208	1828.17	165.65	1992	-	-	365.75	36.87	1623	2528.41	237.33	1064	17	2.45	5	
5: Free MZ	253.41	13.94	116	72.09	13.36	-24	220.47	17.01	58.43	2.47	161	604.39	29.31	158	17.25	0.96	5	
6: MZ diacetate	357.54	33.46	233	193.22	16.17	152	888.48	86.75	221.37	27.87	1074	1660.62	162.61	717	17.67	2.31	12	
7: 1:1 mixture§	568.14	71.81	341	185.39	28.99	82	732.25	110.07	182.23	28.46	556	1668.01	226.64	545	17.5	0.58	4	
8: 1:3 mixture¶	444.64	45.12	311	228.61	71.72	193	889.44	37.75	261.71	70.34	1196	1824.4	173.06	784	18	1.83	6	
¶	<0.001			<0.001			<0.001		<0.001			<0.001						0.883

L, lutein; Z, zeaxanthin; MZ, *meso*-zeaxanthin; CisZ, 13-*cis*-zeaxanthin; TC, total carotenoid (sum of L, Z, MZ and CisZ); YW, yolk weight.

* Repeated-measures ANOVA and paired *t* tests could not be performed because baseline yolk samples could not be paired with their respective 6-week sample, due to the hen housing system used.

† Mean refers to the mean carotenoid content of four eggs; each individual egg yolk sample was analysed without replication.

‡ Free refers to the specified carotenoid in its free form.

§ 1:1 mixture refers to the 1:1 mixture of L and MZ diacetates.

¶ 1:3 mixture refers to the 1:3 mixture of L and MZ diacetates.

|| Statistical significance between groups, tested using *post hoc* one-way ANOVA.

Change in yolk weight and level of pigmentation

Yolk weight did not demonstrate a significant increase over the 6-week study period for individual interventions. However, yolk pigmentation did demonstrate an increase during the study period. At baseline, none of the eggs was in colour category 15 (the highest possible category) but 83 % of the eggs at baseline were in categories 13 or 14, spread comparably across the eight treatment groups. Eggs collected from weeks 3–6, however, showed substantial between-group differences in colour quality. For example, 94 % of eggs from weeks 3–6 in group 4 (Z diacetate), 73 % of eggs from group 7 (1:1 mixture of L:MZ diacetate) and 69 % of eggs from group 2 (L diacetate) were in colour category 15. In contrast, 0 % of eggs from weeks 3–6 in groups 3 (free-form Z) and 5 (free-form MZ) were in colour category 15.

Comparison of free v. esterified supplementation

As can be seen in Fig. 2 and in Table 3, the eggs of hens supplemented with the carotenoid diacetates (groups 2, 4 and 6) all demonstrated greater increases in carotenoid concentrations when compared with their respective free-form carotenoid counterparts. For Z and MZ, the difference in observed responses was statistically significant (free Z *v.* Z diacetate, $P < 0.001$; free MZ *v.* MZ diacetate, $P < 0.001$), whereas this was not the case for L ($P = 0.522$).

Additional carotenoid responses: 13-*cis*-zeaxanthin

It was found that, where appreciable levels of Z and/or MZ were included in the feed, the levels of CisZ, tentatively identified using UV/visible spectroscopy, also increased. This isomer was also present in small amounts in all baseline yolk samples. CisZ was found to correlate highly with total Z at baseline ($R^2 0.781$) and at week 6 ($R^2 0.964$). Given that this *cis*-isomer was not present in the original supplement, yet was present in baseline yolk samples, it seems likely that this *cis*-isomer is a result of natural metabolism of Z and/or MZ by the hen. Data with respect to CisZ at baseline and at week 6 are given in Tables 2 and 3, respectively.

Discussion

The Egg Xanthophyll Intervention Trial represents the first attempt to measure simultaneously the yolk carotenoid response to supplementation with L, Z and MZ in hens. The main aims of this trial were to measure the egg yolk carotenoid response to hen supplementation with L, Z and MZ, and with combinations of these carotenoids, and also to compare the yolk carotenoid response to supplementation with the free and esterified forms of these xanthophylls.

All eight hen intervention groups responded significantly to supplementation in terms of the egg yolk carotenoid concentration. Of interest, in terms of the individual and total carotenoid responses, the Z diacetate intervention group resulted in significantly greater yolk response than the other intervention groups tested, achieving a Z content of 1828 (SD 165) µg/

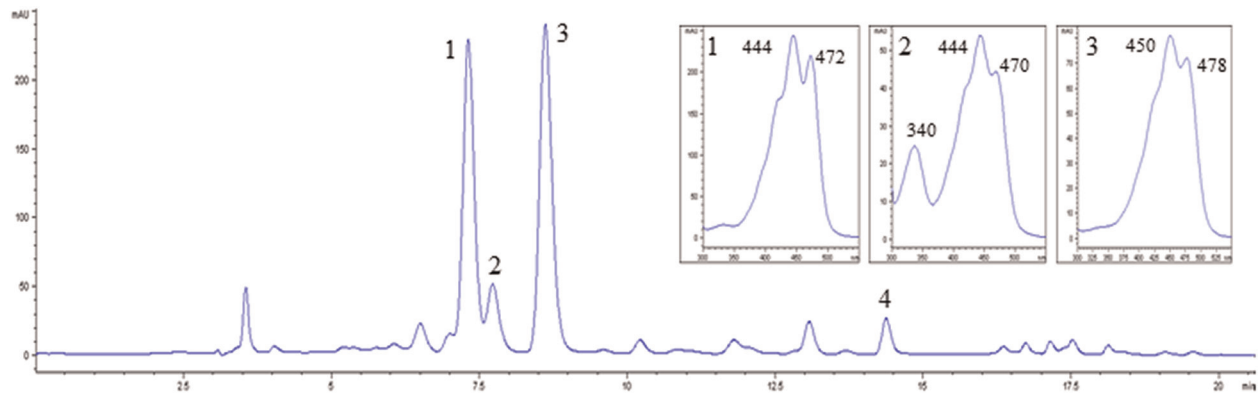


Fig. 3. Chromatogram and absorption spectra of a group 7 (1:1 mixture) yolk extract. Peak 1 = lutein; peak 2 = 13-*cis*-zeaxanthin; peak 3 = total zeaxanthin (mixture of the zeaxanthin and *meso*-zeaxanthin isomers); peak 4 = echinenone as the internal standard. The inset figures show the spectral details and the λ_{\max} of peaks 1, 2 and 3 on the chromatogram. AU, absorbance units.

yolk and a total carotenoid content of 2528 (SD 237) $\mu\text{g}/\text{yolk}$, equating to 1992 and 1064 % increases, respectively. Although there is much more L in blood than Z, comparative studies of xanthophyll absorption found Z to be 30–47 % more efficiently absorbed than L, although these data relate to supplementation with xanthophylls in their free form^(32,33).

Comparison of response to supplementation with the free-form carotenoids *v.* the diacetate carotenoids demonstrates that the esterified forms achieved a significantly greater response in the cases of Z and MZ, but a comparable response in the case of L, suggesting that the bioavailability of Z and MZ is greater in the diacetate form, while that of L is comparable between free and diacetate forms. With respect to colouration of enriched egg yolk, it should be appreciated that formal statistical tests of the ‘group by colour’ contingency table are problematic because of paucity of data (i.e. the conditions for the standard χ^2 are not met, due to the small number of samples and the range of possible colour categories), and while there are additional statistical complications arising from eggs from the same hens being tested at different points in time, the results presented here do suggest that treatment with carotenoids in their diacetate form enhances yolk colour to a greater extent than treatment with these carotenoids in their free form.

Studies by Breithaupt *et al.*⁽³⁴⁾, Wu *et al.*⁽³⁰⁾ and Lai *et al.*⁽³³⁾ report that hens supplemented with free-form *v.* esterified L result in a comparable hen serum carotenoid response and also yolk carotenoid response, suggesting similar bioavailabilities for the free and esterified forms of that carotenoid. However, hen supplementation studies by Galobart *et al.*⁽²⁴⁾ and Hencken⁽³⁵⁾ report greater bioavailability with free (saponified marigold extract) *v.* esterified (non-saponified marigold extract) in terms of yolk carotenoid deposition. The latter finding is consistent with the premise that hens need to first hydrolyse esterified carotenoids prior to absorption in the gastrointestinal tract, while free-form carotenoids can be absorbed in a way that precludes the need for hydrolysis⁽³⁶⁾. The requirement for hydrolysis could, in theory at least, have an adverse impact upon absorption efficiency, and therefore bioavailability, of esterified carotenoid supplements. However, the above studies have made their observations on

the basis of supplementation with esterified marigold extract, which is an ester combination containing 95 % L diester as lutein dipalmitates, myristate-palmitates and palmitate-stearates^(37,38), and not the singular (and lower-molecular-weight) diacetate constituent used in this trial, which has demonstrated an equivalent or superior bioavailability to that of the free-form carotenoids in all cases.

The two mixture groups studied in this trial were 1:1 and 1:3 mixtures of L–MZ diacetate, and these mixtures achieved a statistically comparable total carotenoid response, though the 1:3 mixture of L–MZ diacetate did achieve a higher (but statistically comparable) total carotenoid content than the 1:1 mixture (1824 (SD 173) $\mu\text{g}/\text{yolk}$ (784 % increase) and 1668 (SD 226) $\mu\text{g}/\text{yolk}$ (545 % increase), respectively).

Another carotenoid isomer, which we believe to be CisZ, was identified in the yolk samples at baseline and was observed to increase in parallel with Z and/or MZ when the hens were given Z and/or MZ (either in diacetate or free-form) supplement preparations. This isomer was tentatively identified as CisZ based on its absorbance spectrum, which demonstrated an hypsochromic shift from the normal zeaxanthin spectrum, a large *cis* peak at 340 nm, and lower spectral resolution commonly associated with *cis*-carotenoid spectra⁽³⁹⁾. This isomer also appears in other studies in both supplemented and non-supplemented egg yolks⁽⁴⁰⁾, and appears to be present due to normal metabolic pathways associated with the hens’ digestive and absorbance mechanisms.

This experiment had several limitations which are discussed below. First, as pointed out in our statistical methodology section, for the within-group analysis of changes over time, repeated-measures ANOVA and paired *t* tests could not be performed due to the hen housing system used, which allowed the hens within a specific group to mingle freely, so that eggs from individual hens in each group could not be identified at the different time points. Significant efforts were made in this experiment to provide an environment safe and suitable to control the hens and many different feeding groups, but the inability of this experiment to cage hens individually and collect eggs from individual hens will have introduced variation into the study, as it is known that there are individual differences between birds that were not controlled for in this



experiment. However, we are confident that the customised-built barn for this study provided consistent environmental conditions for each intervention group studied. For example, the barn was newly built, well-sealed from the outside environment, and had no window (to limit unwanted dramatic temperature change). Another limitation of the present study was the omission of a control group during this experiment. However, we know from a follow-on study using the same experimental methodology that there is no significant change in egg yolk xanthophyll concentrations over time when hens are fed a standard diet (i.e. in a control group) (JM Nolan, KA Meagher, AN Howard, R Moran, DI Thurnham and S Beatty, unpublished results).

In conclusion, supplemental L, Z or MZ added to the feed of hens results in egg yolks with significantly higher concentrations of the xanthophyll carotenoids when compared with unsupplemented (baseline) concentrations. Furthermore, the diacetate forms of Z and MZ yield greater responses, in terms of egg yolk concentrations of these carotenoids, than their respective free forms. These findings will inform attempts to develop a novel food designed to increase MP, and its constituent carotenoids in serum, and confer associated health and visual benefits.

Acknowledgements

This project was supported by the Howard Foundation (English Charity reg. no. 285822), Cambridge UK. We would like to acknowledge the hen farmers (Kevin McKenna and Colette McKenna) for supporting this egg experiment on their hen farm.

J.M.N. contributed to the design the trial. J.M.N. was the project manager on the trial and helped with statistical analysis and data interpretation. J.M.N. drafted the final manuscript. K.A.M. contributed to the design of the trial and carried out the experimental procedures, statistical analysis and data interpretation (as part of her post-graduate research study). K.A.M. prepared the initial draft of the manuscript. D.I.T. helped with data interpretation and statistical analysis, and helped to draft the manuscript. S.B. helped to the draft the manuscript and interpret the data. A.N.H. helped with the design of the trial and to draft the manuscript. R.M. contributed to the analytical HPLC work and drafting the paper. All authors have read and approved the final manuscript. J.M.N. is funded by the European Research Council (ERC) under the CREST project (code: 281096) and also holds a Howard Chair at Waterford Institute of Technology in Human Nutrition Research. J.M.N. and S.B. do consultancy work for nutraceutical companies as directors of Nutrasight Consultancy Ltd. D.I.T. is a consultant to the Howard Foundation and receives consulting fees for same. All other authors report no potential conflicts of interest.

References

- Bone RA, Landrum JT, Friedes LM, *et al.* (1997) Distribution of lutein and zeaxanthin stereoisomers in the human retina. *Exp Eye Res* **64**, 211–218.
- Hirsch J & Curcio CA (1989) The spatial resolution capacity of human foveal retina. *Vision Res* **29**, 1095–1101.
- Snodderly DM, Brown PK, Delori FC, *et al.* (1984) The macular pigment. I. Absorbance spectra, localization, and discrimination from other yellow pigments in primate retinas. *Invest Ophthalmol Vis Sci* **25**, 660–673.
- Khachik F, Beecher GR, Goli MB, *et al.* (1992) Separation and identification of carotenoids and their oxidation products in the extracts of human plasma. *Anal Chem* **64**, 2111–2122.
- Sabour-Pickett S, Nolan JM, Loughman J, *et al.* (2012) A review of the evidence germane to the putative protective role of the macular carotenoids for age-related macular degeneration. *Mol Nutr Food Res* **56**, 270–286.
- Sabour-Pickett S, Beatty S, Connolly E, *et al.* (2014) Supplementation with three different macular carotenoid formulations in patients with early age-related macular degeneration. *Retina* **34**, 1757–1766.
- Murray IJ, Makridaki M, van der Veen RL, *et al.* (2013) Lutein supplementation over a one-year period in early AMD might have a mild beneficial effect on visual acuity: the CLEAR study. *Invest Ophthalmol Vis Sci* **54**, 1781–1788.
- Weigert G, Kaya S, Pemp B, *et al.* (2011) Effects of lutein supplementation on macular pigment optical density and visual acuity in patients with age-related macular degeneration. *Invest Ophthalmol Vis Sci* **52**, 8174–8178.
- Loughman J, Nolan JM, Howard AN, *et al.* (2012) The impact of macular pigment augmentation on visual performance using different carotenoid formulations. *Invest Ophthalmol Vis Sci* **53**, 7871–7880.
- Craft NE, Haitema TB, Garnett KM, *et al.* (2004) Carotenoid, tocopherol, and retinol concentrations in elderly human brain. *J Nutr Health Aging* **8**, 156–162.
- Johnson EJ, Vishwanathan R, Johnson MA, *et al.* (2013) Relationship between serum and brain carotenoids, α -tocopherol, and retinol concentrations and cognitive performance in the oldest old from the Georgia Centenarian Study. *J Aging Res* **2013**, 951786.
- Vishwanathan R, Iannaccone A, Scott TM, *et al.* (2014) Macular pigment optical density is related to cognitive function in older people. *Age Ageing* **43**, 271–275.
- Renzi LM, Dengler MJ, Puente A, *et al.* (2014) Relationships between macular pigment optical density and cognitive function in unimpaired and mildly cognitively impaired older adults. *Neurobiol Aging* **35**, 1695–1699.
- Goldberg EM, Ryland D, Gibson RA, *et al.* (2013) Designer laying hen diets to improve egg fatty acid profile and maintain sensory quality. *Food Sci Nutr* **1**, 324–335.
- Stergiadis S, Leifert C, Seal CJ, *et al.* (2014) Improving the fatty acid profile of winter milk from housed cows with contrasting feeding regimes by oilseed supplementation. *Food Chem* **164**, 293–300.
- Perry A, Rasmussen H & Johnson EJ (2009) Xanthophyll (lutein, zeaxanthin) content of fruits, vegetables and corn and egg products. *J Food Comp Anal* **22**, 9–15.
- Maoka T, Arai A, Shimizu M, *et al.* (1986) The first isolation of enantiomeric and meso-zeaxanthin in nature. *Comp Biochem Physiol B* **83**, 121–124.
- Nolan JM, Beatty S, Meagher KA, *et al.* (2014) Verification of meso-zeaxanthin in fish. *J Food Process Technol* **5**, 335.
- Rich GT, Bailey AL, Faulks RM, *et al.* (2003) Solubilization of carotenoids from carrot juice and spinach in lipid phases: I. Modeling the gastric lumen. *Lipids* **38**, 933–945.
- Yeum KJ & Russell RM (2002) Carotenoid bioavailability and bioconversion. *Annu Rev Nutr* **22**, 483–504.
- Courraud J, Berger J, Cristol JP, *et al.* (2013) Stability and bioaccessibility of different forms of carotenoids and vitamin A during *in vitro* digestion. *Food Chem* **136**, 871–877.
- Handelman GJ, Nightingale ZD, Lichtenstein AH, *et al.* (1999) Lutein and zeaxanthin concentrations in plasma after dietary supplementation with egg yolk. *Am J Clin Nutr* **70**, 247–251.



23. Chung HY, Rasmussen HM & Johnson EJ (2004) Lutein bioavailability is higher from lutein-enriched eggs than from supplements and spinach in men. *J Nutr* **134**, 1887–1893.
24. Galobart J, Sala R, Rincón-Carruyo X, *et al.* (2014) Egg yolk color as affected by saponification of different natural pigmenting sources. *J Appl Poult Res* **13**, 328–334.
25. Thurnham DI (2007) Macular zeaxanthins and lutein – a review of dietary sources and bioavailability and some relationships with macular pigment optical density and age-related macular disease. *Nutr Res Rev* **20**, 163–179.
26. Wenzel AJ, Gerweck C, Barbato D, *et al.* (2006) A 12-wk egg intervention increases serum zeaxanthin and macular pigment optical density in women. *J Nutr* **136**, 2568–2573.
27. Meagher KA, Thurnham DI, Beatty S, *et al.* (2013) Serum response to supplemental macular carotenoids in subjects with and without age-related macular degeneration. *Br J Nutr* **110**, 289–300.
28. Thurnham DI, Nolan JM, Howard AN, *et al.* (2015) Macular response to supplementation with differing xanthophyll formulations in subjects with and without age-related macular degeneration. *Graefes Arch Clin Exp Ophthalmol* **253**, 1231–1243.
29. Bowen PE, Herbst-Espinosa SM, Hussain EA, *et al.* (2002) Esterification does not impair lutein bioavailability in humans. *J Nutr* **132**, 3668–3673.
30. Wu L, Huang X, Shi K, *et al.* (2009) Bioavailability comparison of free and esterified lutein for layer hens. *Rev Bras Cienc Avic* **11**, 95–98.
31. Yeum KJ, Booth SL, Sadowski JA, *et al.* (1996) Human plasma carotenoid response to the ingestion of controlled diets high in fruits and vegetables. *Am J Clin Nutr* **64**, 594–602.
32. Thurnham DI, Trémel A & Howard AN (2008) A supplementation study in human subjects with a combination of *meso*-zeaxanthin, (3R,3'R)-zeaxanthin and (3R,3'R,6'R)-lutein. *Br J Nutr* **100**, 1307–1314.
33. Lai S-M, Gray JJ, Flegal CJ, *et al.* (1996) Deposition of carotenoids in eggs from hens fed diets containing saponified and unsaponified oleoresin paprika. *J Sci Food Agric* **72**, 166–170.
34. Breithaupt DE, Weller P & Grashorn MA (2003) Quantification of carotenoids in chicken plasma after feeding free or esterified lutein and capsanthin using high-performance liquid chromatography and liquid chromatography-mass spectrometry analysis. *Poult Sci* **82**, 395–401.
35. Hencken H (1992) Chemical and physiological behavior of feed carotenoids and their effects on pigmentation. *Poult Sci* **71**, 711–717.
36. Tyczkowski JK & Hamilton PB (1986) Lutein as a model dihydroxycarotenoid for the study of pigmentation in chickens. *Poult Sci* **65**, 1141–1145.
37. Rivas JD (1989) Reversed-phase high-performance liquid chromatographic separation of lutein and lutein fatty acid esters from marigold flower petal powder. *J Chromatogr* **464**, 442–447.
38. Breithaupt DE, Wirt U & Bamedi A (2002) Differentiation between lutein monoester regioisomers and detection of lutein diesters from marigold flowers (*Tagetes erecta* L.) and several fruits by liquid chromatography-mass spectrometry. *J Agric Food Chem* **50**, 66–70.
39. Schieber A & Carle R (2005) Occurrence of carotenoid *cis*-isomers in food: technological, analytical, and nutritional implications. *Trends Food Sci Technol* **16**, 416–422.
40. Nimalaratne C, Lopes-Lutz D, Schieber A, *et al.* (2012) Effect of domestic cooking methods on egg yolk xanthophylls. *J Agric Food Chem* **60**, 12547–12552.