

## Fasting plasma zeaxanthin response to *Fructus barbarum* L. (wolfberry; Kei Tze) in a food-based human supplementation trial

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Age-related macular degeneration (AMD) is a common disorder that causes irreversible loss of central vision. Increased intake of foods containing zeaxanthin may be effective in preventing AMD because the macula accumulates zeaxanthin and lutein, oxygenated carotenoids with antioxidant and blue light-absorbing properties. *Lycium barbarum* L. is a small red berry known as *Fructus lycii* and wolfberry in the West, and Kei Tze and Gou Qi Zi in Asia. Wolfberry is rich in zeaxanthin dipalmitate, and is valued in Chinese culture for being good for vision. The aim of this study, which was a single-blinded, placebo-controlled, human intervention trial of parallel design, was to provide data on how fasting plasma zeaxanthin concentration changes as a result of dietary supplementation with whole wolfberries. Fasting blood was collected from healthy, consenting subjects; fourteen subjects took 15 g/d wolfberry (estimated to contain almost 3 mg zeaxanthin) for 28 d. Repeat fasting blood was collected on day 29. Age- and sex-matched controls ( $n$  13) took no wolfberry. Responses in the two groups were compared using the Mann–Whitney test. After supplementation, plasma zeaxanthin increased 2.5-fold: mean values on day 1 and 29 were 0.038 (SEM 0.003) and 0.096 (SEM 0.009)  $\mu\text{mol/l}$  ( $P < 0.01$ ), respectively, for the supplementation group; and 0.038 (SEM 0.003) and 0.043 (SEM 0.003)  $\mu\text{mol/l}$  ( $P > 0.05$ ), respectively, for the control group. This human supplementation trial shows that zeaxanthin in whole wolfberries is bioavailable and that intake of a modest daily amount markedly increases fasting plasma zeaxanthin levels. These new data will support further study of dietary strategies to maintain macular pigment density.

**Macular pigment: AMD: Zeaxanthin: Lutein: *Fructus lycii*: Kei Tze: Wolfberry: Antioxidant: Oxidative stress**

Zeaxanthin is an oxygenated carotenoid (xanthophyll), and a structural isomer of lutein (Edge *et al.* 1997; Moeller *et al.* 2000; Krinsky *et al.* 2003). Both zeaxanthin and lutein are concentrated in the fovea, or ‘yellow spot’, of the eye, and constitute the macular pigment (Landrum & Bone, 2001). In humans, zeaxanthin is located primarily in the central area of the macula, and lutein is found more peripherally (Landrum *et al.* 1999; Bone *et al.* 2000). Thinning of the macular pigment is a common accompaniment to ageing, and is associated with irretrievable loss of central vision, a condition known as age-related macular degeneration (AMD; Beatty *et al.* 1999; Landrum & Bone, 2001; Hyman & Neborsky, 2002). Currently there is no effective treatment for AMD, which is the leading cause of blindness in those aged >65 years (Stokkermans, 2000; Landrum & Bone, 2001).

The underlying causes of AMD are poorly understood, but it is believed that oxidative damage to the lipid-rich retina owing to loss of the protective macular pigment is a key factor (Beatty *et al.* 2000; Landrum & Bone, 2001). The antioxidant properties of lutein and zeaxanthin, along with their ability to absorb blue light, are believed to protect the retina from photo-oxidative damage, reduce light scatter within the eye, and thus maintain visual acuity (Hammond *et al.* 2001; Landrum & Bone, 2001; Benzie, 2003; Krinsky *et al.* 2003; Mozaffarieh *et al.* 2003). Therefore, enhancement of macular pigment by increased dietary

intake of carotenoids is a suggested strategy to delay or prevent AMD and enhance visual function (Hammond *et al.* 1997; Landrum *et al.* 1997; Moeller *et al.* 2000; Stokkermans, 2000). In this regard, lutein has been the focus of several supplementation studies (Landrum *et al.* 1997; Olmedilla *et al.* 2001; Bartlett & Eperjesi, 2003; Bone *et al.* 2003). However, the potential benefit of increased intake of zeaxanthin may be greater than that of lutein, owing to the more centrally located foveal accumulation of zeaxanthin.

The small red berry known variously as *Fructus lycii*, wolfberry, Kei Tze and Gou Qi Zi is the dried, ripe fruit of the plant *Lycium barbarum* L. Wolfberry is rich in zeaxanthin, around 90 % of which is in the form of the ester, zeaxanthin dipalmitate (Lam & But, 1999; Zhou *et al.* 1999; Weller & Breithaupt, 2003). Wolfberry is similar in texture and size to a raisin, and is commonly consumed in China for its pleasant flavour and because of its strong reputation in Chinese culture for being beneficial for vision. The dried berries can be chewed and eaten without further processing, but common practice is to add them to teas, soups and stews. There are no published reports of which we are aware that describe toxic effects or allergic reactions to ingestion of wolfberry.

To date, published studies of plasma zeaxanthin response to supplementation have used purified zeaxanthin or extracts of

wolfberry (Leung *et al.* 2001; Bone *et al.* 2003; Breithaupt *et al.* 2004; Hartmann *et al.* 2004). Leung *et al.* (2001) showed that 6 weeks' supplementation of monkeys ( $n = 3$ ) with a wolfberry extract increased both their plasma and macular zeaxanthin content. Two recently published human trials focused on absorption kinetics of zeaxanthin, one after repeated oral doses of purified, free zeaxanthin (Hartmann *et al.* 2004), and the other on comparing absorption kinetics after a single oral dose of zeaxanthin, extracted from wolfberry, in its free or esterified form (Breithaupt *et al.* 2004). The only food-based human supplementation study we are aware of used egg yolk (Handleman *et al.* 1999). However, ingestion of eggs has the disadvantage of increasing LDL-cholesterol, thereby increasing coronary heart disease risk. Therefore, plasma zeaxanthin in human subjects after dietary supplementation with a natural, cholesterol-free food is of interest. This was the main focus of this study, in which response to 28-d supplementation with wolfberry was determined in a controlled, human intervention trial.

### Methods and materials

This study had the approval of the Ethics Committee of The Hong Kong Polytechnic University, and all procedures involving human subjects complied with the Declaration of Helsinki, as revised in 2000.

This was a single-blinded, placebo-controlled, human intervention trial of 28 d duration and of parallel design. A total of twenty-seven apparently healthy Chinese adults were recruited with their informed consent. The age range of the subjects was 18–48 years (mean 27.6 (SD 9.5)). BMI of volunteers ranged from 17.9 to 24.7 kg/m<sup>2</sup> (mean 20.8 (SD 2.2)). Inclusion criteria included that subjects did not smoke, were not obese, and had no history of glaucoma, cataract, age-related macular degeneration or other retinal diseases. Subjects were excluded if they took regular antioxidant or carotenoid-rich supplements or had a history of serious or chronic disease. Subjects were allocated, on a non-selective basis, but stratified for age and BMI, to the control group ( $n = 13$ ; no supplement) or the study group ( $n = 14$ ; 15 g/d commercially available heat-dried wolfberries ('Rich Nature' brand, kindly supplied by Rich Nature Nutraceutical Labs Inc., Lynwood, USA)). Each volunteer in the study group was provided with a 14-d supply (210 g) of wolfberry, along with instructions on how to prepare their daily supplement. The method of preparation adopted in this study was selected to mimic common usage, but in a standardised manner. In brief, two spoonfuls (a spoon of standard size was supplied) of the berries were added to one cup (around 250 ml) of boiling water and left to stand for around 15 min. The softened berries were then mashed with the back of the spoon, and the berries and water were ingested. Volunteers were requested to take the berries in the evening, shortly after their evening meal. This time was chosen, and the berries fragmented, because carotenoids are more bioavailable in fragmented foods and if taken with fat (Hammond *et al.* 1997; Castenmiller & West, 1998; Bone *et al.* 2000). For the control group, a cup of warm water was taken after dinner each evening for 28 d. After 14 d, members of the study group returned the berry container, and the remaining 14-d supply (210 g) of berries was provided. Compliance was checked by regular telephone calls and e-mail contact, and by checking the contents of the returned container.

Fasting blood was collected, from the antecubital vein, into commercial heparinised blood collection tubes at baseline (day

1) and after 28 d. Blood samples were kept at 4°C and in the dark until separation of plasma, which was within 3 h of collection. Plasma was aliquoted and immediately placed at –70°C. All plasma samples (days 1 and 29 from experimental and control groups) were run in the same batch to minimise analytical variation. Plasma lutein concentrations were also measured in these samples.

Zeaxanthin and lutein were measured simultaneously in extracted plasma using a reversed phase isocratic HPLC method. Pure zeaxanthin and lutein were purchased from Indofine Chemical Company Inc. (Hillsborough, NJ, USA), and purity was checked using HPLC (only one peak was found on the chromatogram of each; results not shown). Absolute ethanol, tetrahydrofuran and hexane (all Chromasolv for HPLC) were from Riedel-de Haen (Sigma-Aldrich Laborchemikalien GmbH, Seelze, Germany), butylated hydroxytoluene (BHT) was from Sigma (St. Louis, MO, USA), ascorbic acid was from E. Merck (Darmstadt, Germany) and acetonitrile (HiperSolv for HPLC) was from BDH Laboratory Supplies (Poole, UK). Milli-Q water was used (Millipore Corp. Billerica, MA, USA). The chromatography system included an Alliance 2695 Separation Module with temperature-controlled column chamber and autosampler (Waters, Milford, MA, USA), Waters 2996 Photodiode Array Detector and Waters Empower Pro PDA software (version 5.00.00.00), a reversed-phase Luna C<sub>18</sub> analytical column (5 µm, 250 × 4.6 mm i.d.; Phenomenex, Torrance, CA, USA) and a cartridge guard column (Security Guard, C<sub>18</sub>, cartridge 4 × 3.0 mm i.d., from Phenomenex). The mobile phase was 0.1% (w/v) BHT in acetonitrile, which was filtered and degassed through a Millipore membrane (type GV, pore size 0.22 µm, Millipore Corp.) before use. Detection was at 450 nm. On the basis of preliminary work, the flow rate was time-programmed at two speeds, 1.0 ml/min for 0–12 min post-injection, and 2.5 ml/min for 12–60 min. The columns worked under room temperature but the autosampler cooling chamber was at 4°C.

Zeaxanthin and lutein are sensitive to light and oxygen. To minimise degradation, all procedures for preparing calibrators, controls and test samples were performed in dim light, and evaporation of extracts was under nitrogen. In addition, two antioxidants were added (0.1% w/v ascorbic acid in aqueous phase and 0.1% w/v BHT in non-aqueous phase) to minimise oxidative losses during sample processing and analytical procedures, and aluminium foil was used to wrap sample containers to protect from direct illumination of light. Stock standards of pure zeaxanthin and lutein were prepared in 0.1% (w/v) BHT in tetrahydrofuran. On the day of use, these were diluted in 0.1% (w/v) BHT in ethanol and mixed, immediately prior to use, to provide working calibrators over the expected concentration range.

The extraction procedure was modified from that described by Aebischer *et al.* (1999). For calibrators, 200 µl of pooled heparinised plasma (in a 1.5 × 10 cm glass, screw-capped test-tube) was firstly mixed with 200 µl of Milli-Q water containing ascorbic acid (0.1% w/v). To this solution, 20 µl of one of the calibrators and 380 µl of 0.1% (w/v) BHT–ethanol were added. The mixture was vortexed for 30 s., following addition of 800 µl of hexane containing BHT (0.1% w/v), the tubes (covered with aluminium foil) were placed on a horizontal mixer (Denley Spiramix 5; Denley Instruments Ltd, Billingham, UK) for 30 min. The suspension was then centrifuged in a Jouan CR4-12 centrifuge (Jouan Ltd, France) at 2000 rpm, 4°C, for 10 min. A 400 µl aliquot of hexane (upper layer) containing the extracted zeaxanthin was

withdrawn and evaporated to dryness at  $\sim 40^{\circ}\text{C}$  under a slow stream of nitrogen. The residue was then vortexed with  $20\ \mu\text{l}$  of tetrahydrofuran (containing 0.1% w/v BHT), followed by addition of  $80\ \mu\text{l}$  of mobile phase. The mixture was again vortexed to re-dissolve the residue. These extraction procedures were performed for all calibrators on the day of use. For extraction of test plasma or control,  $200\ \mu\text{l}$  of plasma or control (zeaxanthin-spiked pooled plasma) were mixed with  $200\ \mu\text{l}$  of 0.1% (w/v) ascorbic acid–water and  $400\ \mu\text{l}$  of 0.1% (w/v) BHT–ethanol in the first step, with the rest of the extraction as above. Finally,  $25\ \mu\text{l}$  of each extracted calibrator, sample or control was injected into the HPLC system. Peak areas of zeaxanthin and lutein were recorded from each chromatogram. By plotting the peak areas against concentrations, calibration curves were constructed. The concentration of each sample was calculated by dividing the peak area in the sample by the slope of the appropriate calibration curve.

The linear range was determined by direct injections of standards at various concentrations. Linearity of response was assessed from the coefficient of correlation ( $r$ ) of the regression line. Six replicates of each calibrator at various concentrations were used to assess within-day precision. The slopes from assays on 6 d (consecutive) were used to assess between-day precision. Precision was also assessed using separate extractions and injections of pooled plasma and pooled plasma spiked with zeaxanthin and lutein. To determine the limit of detection (LD), the signal to noise ratio limit was at least 2:1. The LD is the smallest amount that can be differentiated from zero, and was calculated as  $\text{mean} + 3\text{SD}$  of ten blank readings. For recovery studies, pooled plasma was spiked with zeaxanthin and lutein, and native ( $n\ 4$ ) and spiked plasma samples ( $n\ 4$ ) were then extracted and assayed. By comparing results on samples with those obtained with direct injections of the same (spike) amounts of pure zeaxanthin and lutein ( $n\ 4$  each), recoveries were assessed.

Using this method, the peaks for lutein and zeaxanthin were well resolved, and both eluted within 12 min (Fig. 1).  $\beta$ -Carotene eluted very slowly, taking more than 2 h at a flow rate of 1.0 ml/min to appear. As this  $\beta$ -carotene peak may interfere and overlap with peaks of interest in subsequent samples, sample injection intervals must be long enough to allow column washout. To avoid overly long injection intervals, the flow rate was programmed to increase to 2.5 ml/min once lutein and zeaxanthin had eluted (i.e. after 12 min). With this setting,  $\beta$ -carotene eluted at around 55 min, and the auto-injection interval was set to 60 min, giving an acceptable throughput. Within-day coefficients of variation (CVs) for zeaxanthin were 4% at  $0.01\ \mu\text{mol/l}$  and 4% at  $0.04\ \mu\text{mol/l}$  ( $n\ 6$ ); between-day CVs were 7% at  $0.018\ \mu\text{mol/l}$  and 6% at  $0.03\ \mu\text{mol/l}$  ( $n\ 6$ ). Within-day CVs for lutein were 7% at  $0.35\ \mu\text{mol/l}$  and 3% at  $0.87\ \mu\text{mol/l}$  ( $n\ 6$ ). Between-day CVs for lutein were 9% at  $0.35\ \mu\text{mol/l}$  and 5% at  $0.70\ \mu\text{mol/l}$  ( $n\ 6$ ). Recovery of  $0.009\ \text{nmol}$  zeaxanthin was 96.1% ( $n\ 4$ ). Dose–response for zeaxanthin is shown in Fig. 2. Linearity was maintained at least up to  $1.75\ \mu\text{mol/l}$  ( $r\ 0.9998$ ) zeaxanthin. The LD was 0.5 pmol; under the assay conditions used this is equivalent to an LD of  $0.0024\ \mu\text{mol/l}$  for plasma zeaxanthin (also for lutein). The sensitivity of the analysis, or minimum detectable difference, was estimated based on the  $\text{mean} + 3\text{SD}$  of peak areas obtained by six replicate extractions and injections of pooled plasma, and was  $0.0027\ \mu\text{mol/l}$ .

Carotenoids are carried in plasma within lipoproteins and are affected by lipid levels, therefore both total (as  $\mu\text{mol/l}$ ) and

lipid-standardised (as nmol zeaxanthin (or lutein) per mmol total cholesterol plus triacylglycerol) concentrations are presented. Plasma total cholesterol and triacylglycerol were measured using commercial test kit methods (Biosystems S.A., Spain) on a Cobas Fara centrifugal analyser (Roche, Basel, Switzerland).

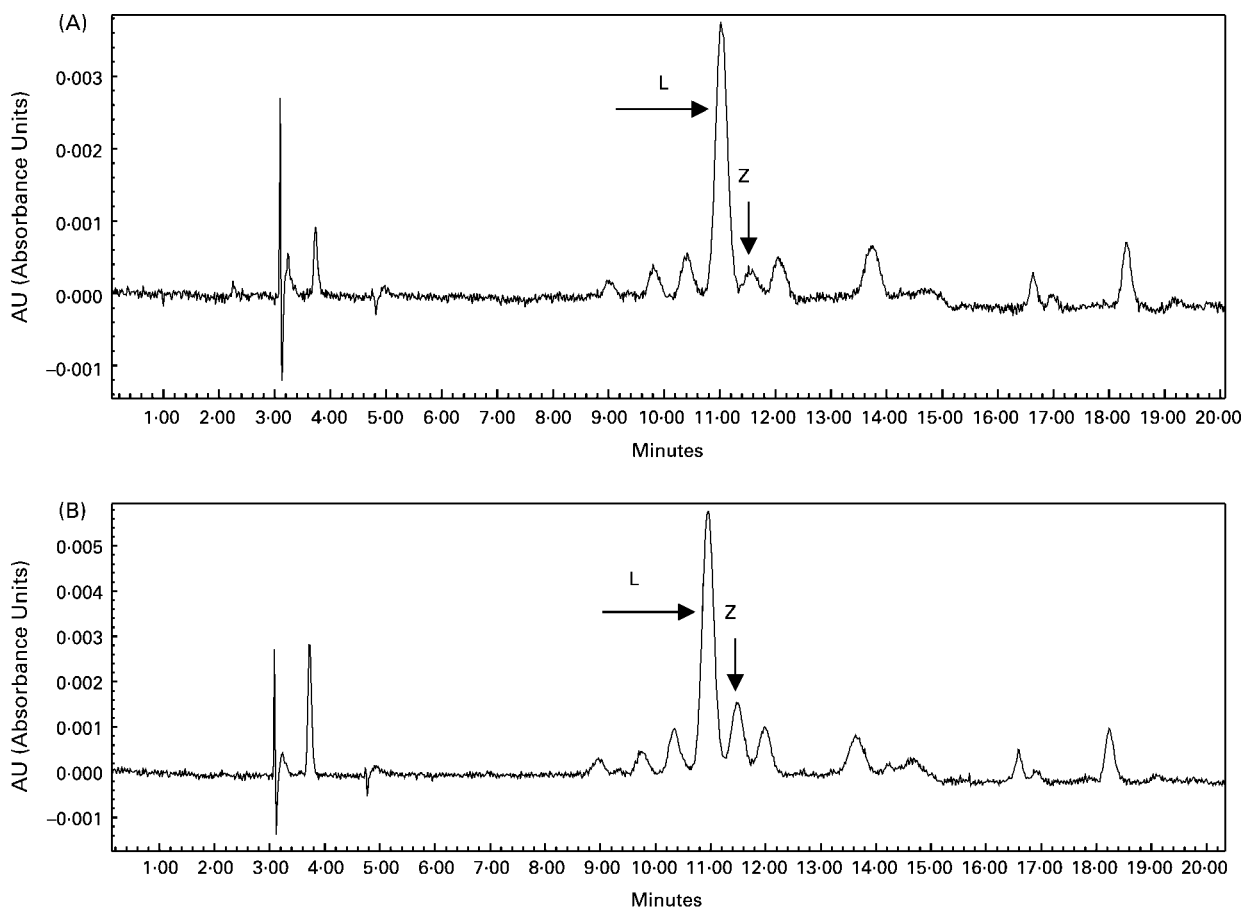
To explore the dose of zeaxanthin supplied by the berries used in the supplementation study, a weighed sample of berries was extracted, zeaxanthin esters were hydrolysed and the total zeaxanthin content estimated by HPLC. Berries were homogenised in 0.5 ml of 6 M-NaOH, and 1 ml of absolute ethanol containing 0.1% (w/v) BHT was added. The mixture was sonicated for 3 min, followed by incubation at  $50^{\circ}\text{C}$  for 3 h for hydrolysis of zeaxanthin esters, after which 0.5 ml of 6 M-HCl was added and the pH of the mixture adjusted to 7. Two ml of hexane containing 0.1% (w/v) BHT was added for extraction of carotenoids, with mixing (on a Denley Spiramix 5) for 30 min, after which the suspension was centrifuged in a Jouan CR4-12 centrifuge (Jouan Ltd) at 2000 rpm,  $4^{\circ}\text{C}$ , for 10 min. One ml of the hexane layer was removed and evaporated to dryness under a stream of nitrogen at  $40^{\circ}\text{C}$ . The residue was re-dissolved in  $20\ \mu\text{l}$  of tetrahydrofuran containing 0.1% (w/v) BHT and  $80\ \mu\text{l}$  of 100% acetonitrile containing 0.1% (w/v) BHT, and  $5\ \mu\text{l}$  of the redissolved extract was injected into the HPLC system for determination of zeaxanthin and lutein. This procedure was performed once only, was not intended to be a definitive assessment of the zeaxanthin content of wolfberry, but aimed to furnish an approximation of the daily dose of zeaxanthin in the berries ingested.

For statistical analysis, Graphpad Prism was used (version 3.0, San Diego, USA). The pre-versus post- data were analysed using the Wilcoxon matched pairs test to compare data on days 1 and 29 for each subject. The Mann–Whitney test was used to compare responses to wolfberry and placebo;  $P < 0.05$  was considered significant.

## Results

Demographic information on the control and study groups is summarised in Table 1. There were no significant differences in age, weight or BMI between the two groups. Day 29 samples were not available for three subjects in the control group, and for one subject in the study group; data are presented for ten control subjects and thirteen supplemented subjects.

The concentration of zeaxanthin (after hydrolysis) in the berries extracted was estimated at  $194\ \mu\text{g/g}$ . Based on this, the 15 g supplement provided an additional dietary intake of zeaxanthin of approximately 3 mg/day. The lutein content of the berries, which was measured at the same time as zeaxanthin, was much lower, at  $5.8\ \mu\text{g/g}$ . Therefore the daily berry intake would have provided  $< 0.1\ \text{mg/d}$  of additional lutein. After 28-d supplementation, the lutein concentration of fasting plasma had not changed significantly. However, there was a significant ( $P < 0.01$ ) and marked increase in both the total and the lipid-standardised plasma zeaxanthin concentrations (Table 2). One subject showed a poor response, with a pre–post difference of  $0.007\ \mu\text{mol/l}$  in plasma zeaxanthin. This subject had the highest pre-supplementation fasting plasma zeaxanthin concentration, but no significant correlation overall was seen between pre-supplementation plasma zeaxanthin concentrations and the magnitude of individual responses ( $r\ -0.517$ ;  $P > 0.05$ ). The data for this subject are included in Table 2.



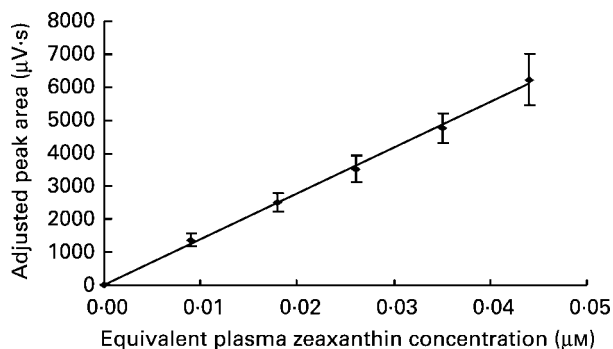
**Fig. 1.** Chromatogram of extracted plasma from a single volunteer, pre- (A) and post- (B) supplementation. Peaks of lutein (L) and zeaxanthin (Z) (identified by comparison with injection of pure lutein and zeaxanthin) are arrowed.

## Discussion

The human diet is estimated to supply 1.3–3 mg/d of lutein and zeaxanthin combined (Sommerburg *et al.* 1998). However, as most xanthophyll-containing foods, such as corn, spinach, broccoli, orange and kiwi fruit, contain four to seven times more lutein than zeaxanthin, dietary zeaxanthin intake is usually low and fasting plasma levels of zeaxanthin are generally  $<0.1 \mu\text{mol/l}$  (Bone *et al.* 2003; Breithaupt *et al.* 2004; Hartmann *et al.* 2004). To date, lutein has been the xanthophyll of interest in most AMD-related supplementation studies (Sommerburg *et al.*

1998; Johnson *et al.* 2000; Landrum & Bone, 2001; Bone *et al.* 2003). Data on plasma and macular pigment density changes following zeaxanthin supplementation are scarce, but are of interest in relation to the more specific accumulation of zeaxanthin in the central macula of humans (Bone *et al.* 2000).

The study of Hartmann *et al.* (2004) reported that fasting plasma zeaxanthin concentrations increased around 4-fold and 20-fold, from an arithmetic mean of  $0.05 \mu\text{mol/l}$  at baseline to a geometric mean of  $0.20$  and  $0.92 \mu\text{mol/l}$  after 6 weeks of supplementation with, respectively, 1 and 10 mg/d of pure, synthetic, non-esterified zeaxanthin ( $n = 10$  for each dose). Absorption kinetics were monitored for 24 h post-ingestion of both doses, and peak levels were seen at 10–15 h. The 0–24 h area under the curve (AUC) for the 10 mg dose was almost six times higher than that of the 1 mg dose after a single dose and after 6 weeks of daily dosing, but individual responses were highly variable (Hartmann *et al.* 2004). Plasma levels reached  $>90\%$  of



**Fig. 2.** Representative calibration curve for zeaxanthin: each point is mean of four readings (separate extractions) with  $\pm 2\text{SD}$  error bars shown.

**Table 1.** Demographics of study ( $n = 14$ ) and control ( $n = 13$ ) groups

	Study group		Control group	
	Mean	SEM	Mean	SEM
Age range (years)	18–44		18–48	
Age (years)	28.0	2.68	27.2	2.54
Height (m)	1.59	0.016	1.62	0.025
Weight (kg)	51.39	1.76	57.68	2.92

**Table 2.** Plasma zeaxanthin and lutein in fasting samples collected pre- and post-28-d supplementation with 15 g/d wolfberry (*Fructus lycii*; Kei Tze; Gou Qi Zi) or placebo

	Placebo (n 10)				Wolfberry (n 13)							
	Day 1		Day 29		Response to placebo		Day 1		Day 29		Response to wolfberry	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Plasma zeaxanthin (µmol/l)	0.0377	0.0033	0.0433	0.0031	0.00562	0.0033	0.0382	0.0034	0.0960 <sup>a</sup>	0.0096	0.0578 <sup>b</sup>	0.011
Lipid-standardised plasma zeaxanthin (nmol/mmol TC + Tg)	7.01	0.804	8.49	0.831	1.48	0.797	7.58	0.638	18.1 <sup>a</sup>	1.51	10.48 <sup>b</sup>	1.74
Plasma lutein (µmol/l)	0.144	0.018	0.170	0.024	0.0256	0.014	0.164	0.021	0.164	0.022	0.0001	0.013
Lipid-standardised plasma lutein (nmol/mmol TC + Tg)	27.2	3.83	33.3	4.82	6.15	2.34	32.2	4.00	31.4	4.05	-0.80	3.74

TC + Tg, total cholesterol plus triacylglycerol.

<sup>a</sup>Significantly different from pre-supplementation level,  $P < 0.01$  (Wilcoxon matched pairs test).<sup>b</sup>Significantly different from response to placebo,  $P < 0.01$  (Mann–Whitney test).

steady-state concentration after around 17 d of supplementation, and levels decreased rapidly when the supplementation was stopped (Hartmann *et al.* 2004). This rapid decrease in plasma zeaxanthin after discontinuation of dosing was seen also by Bone *et al.* (2003), who reported plateau fasting levels of 0.56 and 0.48 µmol/l in each of two subjects who had been supplemented with 30 mg/d of crystalline, unesterified zeaxanthin for several weeks. This represented a 5–6-fold increase from baseline. In the study by Breithaupt *et al.* (2004), twelve subjects took (separately) two zeaxanthin-based extracts of wolfberry. Plasma levels after ingestion of both extracts plateaued at about 9 h post-ingestion, and the increase in plasma zeaxanthin averaged 0.02 µmol/l after the unesterified zeaxanthin extract, and about 0.06 µmol/l after ingestion of the same amount (5 mg) of the esterified (dipalmitate) extract. The plasma zeaxanthin response in terms of 0–24 h AUC was significantly higher, but was also much more variable, following ingestion of the esterified extract, but the speed of response to both types appeared similar (Breithaupt *et al.* 2004). The authors suggested that the greater variation of response following ingestion of the esterified form may be due to variation in intestinal lipase activity, and that the dipalmitate form may be more bioavailable than the more polar, non-esterified form because of more effective micelle formation. It is of interest that no increase in plasma levels of zeaxanthin esters was seen following ingestion of the esterified form of zeaxanthin (Breithaupt *et al.* 2004).

In plant foods, zeaxanthin is found mainly in the esterified form (Lam & But, 1999; Zhou *et al.* 1999; Krinsky *et al.* 2003; Weller & Breithaupt, 2003). Zeaxanthin is found in relatively high concentration in egg yolk (210 µg/yolk), but this is mainly in the unesterified form. The petals of some flowers, such as marigold, are also a rich source of zeaxanthin (500 µg/100 mg) (Handleman *et al.* 1999; Olmedilla *et al.* 2001). Marigold extracts are available as supplements, but flowers are not a common component of the human diet. Wolfberry is rich in zeaxanthin dipalmitate, but estimates of berry content vary depending on grade and source of the berries. Lam & But (1999) reported a total zeaxanthin content of between 1.1 and 4.3 mg/100 g, with an average of 2.4 mg/100 g, in five different samples of wolfberry. Leung *et al.* (2001) stated that values of 30 mg/100 g have been found. Weller & Breithaupt (2003) reported a much higher content (82 mg/100 g). We performed an exploratory study of zeaxanthin content of the berries used in this study and, based on the 19.4 mg/100 mg found, estimate the dose supplied in the 15 g daily dose of berries to be around 3 mg zeaxanthin.

Spinach and corn are also rich in lutein and zeaxanthin. However, increased intake of cooked spinach and corn did not lead to an increase in plasma zeaxanthin concentrations (Bone *et al.* 2000; Mozaffarieh *et al.* 2003). Clearly, the issue of bioavailability of zeaxanthin from different foods, as well as in different purified forms, is an important one. It is known that ingestion of fat is needed to facilitate absorption of carotenoids, and this may help explain the high bioavailability of zeaxanthin in egg yolk (Handleman *et al.* 1999). However, it is possible that zeaxanthin is less well absorbed and distributed than lutein (Castenmiller & West, 1998; Bone *et al.* 2003). The study of Bone *et al.* (2003) showed that the relative change in plasma lutein was twice that of zeaxanthin in the same subject supplemented with 30 mg/d lutein and, separately, zeaxanthin, and the plateau supplementation-induced plasma zeaxanthin level was less than one-third that of lutein (Bone *et al.* 2003). However, as pointed out by the authors, the low response to zeaxanthin may have been due to differences in the formulations used,

as the lutein used was esterified and the zeaxanthin was unesterified (Bone *et al.* 2003).

Increased intake of some zeaxanthin-rich foods does increase plasma zeaxanthin concentration. Supplementation with egg yolk was reported (Handleman *et al.* 1999) to increase plasma lutein and zeaxanthin by, respectively, 28 % and 142 %. However, LDL-cholesterol increased also, by approximately 10 %. This increase is estimated to increase long-term heart disease risk by 14 % in men with baseline cholesterol concentrations about 5.2 mmol/l, the upper limit of the desirable range; the same increase in men with higher cholesterol concentrations would increase risk of heart disease to a greater extent (Verschuren *et al.* 1995). Therefore an egg yolk-based dietary strategy to increase plasma zeaxanthin cannot be recommended, and an alternative, cholesterol-free, food source is desirable. In this current study, we demonstrate a mean post-supplementation level of 0.096  $\mu\text{mol/l}$ , a 250 % increase, in fasting plasma zeaxanthin after 4 weeks of supplementation with whole wolfberries estimated to supply  $\sim 3$  mg/d total zeaxanthin, more than is contained in a dozen egg yolks, and no deleterious effects on plasma lipids were seen (results not shown).

AMD is the leading cause of blindness in the elderly, is more common in smokers, and is associated with thinning of the macular pigment (Hyman & Neborsky, 2002; Krinsky *et al.* 2003). There are observational data to support the link between increased intake of xanthophyll-rich foods and lower risk of AMD, but cause and effect relationships are still tenuous (Krinsky *et al.* 2003). It is believed that the beneficial effects of increased intake of lutein and zeaxanthin are mediated by increased macular pigment. A significant increase in macular pigment in association with increased plasma lutein has been reported in human subjects after 4 or more weeks of supplementation with lutein supplements or lutein-rich food, but not all studies find this (Hammond *et al.* 1997; Landrum *et al.* 1997; Sommerburg *et al.* 1998; Moeller *et al.* 2000; Mares-Perlman *et al.* 2002; Bartlett & Eperjesi, 2003; Bone *et al.* 2003; Mozaffarieh *et al.* 2003). This may be

owing to the type of supplement or food used, and to the duration of the dosing. It has been suggested also that there may be 'responders' and 'non-responders' to increased xanthophyll intake, and published data show very variable responses, both in time and magnitude (Hammond *et al.* 1997; Leung *et al.* 2001; Bone *et al.* 2003; Breithaupt *et al.* 2004; Hartmann *et al.* 2004). In the study by Leung *et al.* (2001), serum zeaxanthin and lutein concentrations in rhesus monkeys ( $n = 3$ ) were measured before and after 6 weeks of supplementation with 2.2 mg zeaxanthin/d in the form of a wolfberry extract. Serum zeaxanthin concentrations were reported to increase 2.5-fold compared with basal levels, and the average zeaxanthin content in the maculae of the treated animals was twice that of control animals. However, examination of the data (Leung *et al.* 2001) shows that one of the three animals fed an extract of wolfberry showed no increase in plasma zeaxanthin. Intriguingly, though having the lowest pre- and post-supplementation plasma zeaxanthin levels, this animal was shown to have the highest macular content of zeaxanthin at the end of the study. The significance of this finding is not clear. Furthermore, contrary to humans who accumulate zeaxanthin in the central area of the macula, the species used in the study, rhesus monkeys (*Macaca mulatta*), concentrate lutein centrally (Leung *et al.* 2001).

In human studies (Hammond *et al.* 1997; Landrum *et al.* 1997; Bone *et al.* 2003) increased macular pigment density has been reported with lutein and zeaxanthin supplementation. Interestingly, data from a small ( $n = 2$ ) human study of high-dose zeaxanthin supplementation (30 mg/d; Bone *et al.* 2003) showed that the increase in macular pigment lagged behind that in plasma zeaxanthin. Plateau plasma levels of zeaxanthin were seen after about 30 and 10 d of supplementation for the two subjects studied, but macular pigment did not begin to increase until 1–2 weeks after these times. However, thereafter the macular pigment increased in an approximately linear way. In the single subject who continued with supplementation to 120 d, the macular pigment density plateaued at about 20 weeks after the

**Table 3.** Summary of human trials of fasting plasma response to zeaxanthin supplementation

Supplement type, dose and duration	<i>n</i>	Post-supplementation fasting plasma zeaxanthin in absolute ( $\mu\text{mol/l}$ ) and relative terms	Comment	Reference
Pure, synthetic, unesterified zeaxanthin; 1 mg/d and 10 mg/d for 42 d	10	Mean 0.20 (SD 0.07) at 1 mg dose ( $\times 4$ increase) and 0.92 (SD 0.28) at 10 mg dose ( $\times 20$ increase)	Parallel study; dose-response seen ( $\times 10$ dose gave $\times 5$ response)	Hartmann <i>et al.</i> (2004)
Crystalline, unesterified zeaxanthin; 30 mg/d for 60 d (one subject) and 120 d (one subject)	2	Individual values: 0.48 ( $\times 5$ increase); 0.56 ( $\times 6$ increase)	Plateau reached for one subject at $\sim 10$ d; $\sim 30$ d for the other; zeaxanthin response lower than to same dose of esterified lutein	Bone <i>et al.</i> (2003)
1.5 egg yolks/d supplying $\sim 280$ $\mu\text{g/d}$ zeaxanthin ( $>90\%$ in unesterified form) for 32 d (4.5 weeks)	11	Mean 0.105 (SD 0.023) with corn oil diet ( $\times 2$ increase); 0.116 (SD 0.027) with beef tallow diet ( $\times 2.5$ increase)	Cross-over study; zeaxanthin in egg yolk highly bioavailable; 8–11 % increases seen in plasma LDL	Handleman <i>et al.</i> (1999)
15 mg/d wolfberries for 28 d; whole berry supplement estimated to provide $\sim 3$ mg/d esterified zeaxanthin	27 (test group, $n = 14$ ; control group, $n = 13$ )	Mean 0.096 (SD 0.034) ( $\times 2.5$ increase) in test group	Controlled intervention trial; parallel design; no significant correlation seen between fasting zeaxanthin at entry and magnitude of response	This study

supplementation had begun. Interestingly, this was 20 d after supplementation was stopped, and when plasma levels had decreased to pre-supplementation levels. The speed of both plasma and macular changes in one of the subjects was faster than the other (Bone *et al.* 2003). Similar findings were reported for lutein (Landrum *et al.* 1997). This is congruent with the concept that plasma xanthophyll supply helps determine macular delivery and enrichment. It is interesting to note also that, while the plasma concentration decreased rapidly when supplementation was discontinued, the macular pigment was maintained at its higher, supplementation-related level for the following 4 months (Bone *et al.* 2003). The authors suggested that this could indicate that continued high-dose supplementation may not be necessary for maintenance of the macular pigment, and that an initial loading followed by a low maintenance dose may be adequate (Bone *et al.* 2003). The recent finding that the esterified form of zeaxanthin (zeaxanthin dipalmitate) induces a greater plasma response than the unesterified form (Breithaupt *et al.* 2004) suggests that the increases in macular pigment seen after 30 mg/d of unesterified zeaxanthin (Bone *et al.* 2003) could be induced by a smaller dose of esterified zeaxanthin. This remains to be confirmed.

When data from different studies were pooled, significant correlation was seen between the dosage of lutein and the plateau plasma concentration attained, and between this and the rate of increase in macular pigment density (Bone *et al.* 2003). For zeaxanthin, there is evidence that greater doses induce a greater plasma response (Hartmann *et al.* 2004), but individual response varies widely. At this time it does not appear that the magnitude of plasma response is determined or limited by the pre-existing plasma concentration *per se*. Data in relation to dose of zeaxanthin and plasma levels attained after a period of supplementation are summarised in Table 3. Insufficient data exist at this time to investigate correlation between increases in plasma zeaxanthin and macular pigment density response, and further work is needed in this area.

In conclusion, results of this controlled, food-based, supplementation trial show that short-term supplementation with a modest daily dose (15 mg) of whole wolfberries taken in a similar way as in normal dietary practice markedly increased zeaxanthin in fasting plasma of healthy subjects. Therefore, wolfberry offers an inexpensive, effective and safe whole food dietary strategy to increase plasma zeaxanthin concentration. The implications for benefit of wolfberry intake in terms of ocular health are strong. Longer-term supplementation studies are now needed in regard to the potential for wolfberry to increase macular pigment density and help maintain visual acuity in our ageing population through its effect on plasma zeaxanthin concentration.

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