British Journal of Nutrition (2004), **91**, 707–713
DOI: 10.1079/BJN20041105

Comparison of plasma responses in human subjects after the ingestion of 3R,3R′-zeaxanthin dipalmitate from wolfberry (*Lycium barbarum*) and non-esterified 3R,3R′-zeaxanthin using chiral high-performance liquid chromatography

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(Received 24 November 2003 – Revised 23 January 2004 – Accepted 30 January 2004)

Age-related macular degeneration (AMD) is one of the most common eye diseases of elderly individuals. It has been suggested that lutein and zeaxanthin may reduce the risk for AMD. Information concerning the absorption of non-esterified or esterified zeaxanthin is rather scarce. Furthermore, the formation pathway of *meso* (3R,3′S)-zeaxanthin, which does not occur in plants but is found in the macula, has not yet been identified. Thus, the present study was designed to assess the concentration of 3R,3R′-zeaxanthin reached in plasma after the consumption of a single dose of native 3R,3′R-zeaxanthin palmitate from wolfberry (*Lycium barbarum*) or non-esterified 3R,3′R-zeaxanthin in equal amounts. In a randomised, single-blind cross-over study, twelve volunteers were administered non-esterified or esterified 3R,3′R-zeaxanthin (5 mg) suspended in yoghurt together with a balanced breakfast. Between the two intervention days, a 3-week depletion period was inserted. After fasting overnight, blood was collected before the dose (0 h), and at 3, 6, 9, 12, and 24 h after the dose. The concentration of non-esterified 3R,3′R-zeaxanthin was determined by chiral HPLC. For the first time, chiral liquid chromatography–atmospheric pressure chemical ionisation-MS was used to confirm the appearance of 3R,3′R-zeaxanthin in pooled plasma samples. Independent of the consumed diet, plasma 3R,3′R-zeaxanthin concentrations increased significantly ($P=0{\cdot}05$) and peaked after 9–24 h. Although the concentration curves were not distinguishable, the respective areas under the curve were distinguishable according to a two-sided F and t test ($P=0{\cdot}05$). Thus, the study indicates an enhanced bioavailability of 3R,3′R-zeaxanthin dipalmitate compared with the non-esterified form. The formation of *meso*-zeaxanthin was not observed during the time period studied.

3R,3′R-zeaxanthin: Zeaxanthin esters: Plasma carotenoid response: Chiral analysis: Liquid chromatography–atmospheric pressure chemical ionisation-mass spectrometry

Zeaxanthin (β,β-carotene-3,3′-diol) is a xanthophyll widely distributed in fruits and vegetables. Data concerning the zeaxanthin concentration of plants have been specified by several authors (Sommerburg *et al.* 1998; Murkovic *et al.* 2000; Breithaupt & Bamedi, 2001). Recently, a study investigating zeaxanthin esters in plants with potential use for zeaxanthin oleoresin production was published (Weller & Breithaupt, 2003). During the last decade, lutein and zeaxanthin attracted attention as both xanthophylls were found to be specifically located in the human macula (for example, Bernstein *et al.* 2001; Landrum & Bone, 2001). Relying on epidemiological data, it is anticipated that a high serum concentration of lutein and zeaxanthin could protect from age-related macular degeneration and age-related cataract formation (for example, Mares-Perlman *et al.* 2002). The enrichment of the daily human diet with zeaxanthin-containing plants has been known for a long time; for example, wolfberries (*Lycium barbarum*; Gou Qi Zi), small red berries commonly used in home cooking in China, were traditionally used in Chinese herbal medicine for the improvement of visual acuity. The main phytochemical found in this plant source, which is regarded as the active component, is zeaxanthin dipalmitate, a diester formed from zeaxanthin and palmitic acid (Lam & But, 1999; Zhou *et al.* 1999; Barua, 2001). Leung *et al.* (2001) used rhesus monkeys to study the serum and tissue levels of zeaxanthin and lutein after feeding Gou Qi Zi extracts.

Due to the two chiral centres at the carbon atoms C_3 and C_3', the positions of the secondary hydroxyl groups, zeaxanthin theoretically exists in three stereoisomeric forms: 3R,3′R-, 3S,3′S- and 3R,3′S-zeaxanthin; the latter is also referred to as *meso*-zeaxanthin. In plants, usually 3R,3′R-zeaxanthin occurs as the only form, whereas in shrimp, fish, and reptiles all enantiomers can be present in parallel (Maoka *et al.* 1986). Xanthophylls extracted from human

Abbreviations: APcI, atmospheric pressure chemical ionisation; AUC, area under the curve; LC, liquid chromatography.

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retina, analysed after derivatisation on reversed-phase columns, showed that only two forms were present: 3R,3′R- and *meso*-zeaxanthin. Besides zeaxanthin enantiomers, lutein was detected in its native 3R,3′R,6′R form (Bone *et al.* 1993). The authors postulated that *meso*-zeaxanthin, which is not of dietary origin, is generated from 3R,3′R,6′R-lutein present in the retina of the eye. However, the process is not yet sufficiently understood. Khachik *et al.* (2002), who found no *meso*-zeaxanthin in human plasma and liver, postulated double-bond isomerisation as the metabolic pathway for the generation of the *meso* form. Landrum & Bone (2001) suggested 3′-oxolutein as an intermediate in the conversion mechanism within the retina. However, the potential formation of *meso*-zeaxanthin after the ingestion of 3R,3′R-zexanthin-enriched diets has not been studied so far.

Dietary xanthophyll esters are hydrolysed during the digestion process before absorption occurs (Wingerath *et al.* 1995). Data comparing the resorption characteristics of esterified and non-esterified zeaxanthin are however not yet available. Comparable work has been done on lutein and its esters (Bowen *et al.* 2002) and on β-cryptoxanthin (Breithaupt *et al.* 2003). Pérez-Gálvez *et al.* (2003) recently reported that capsanthin from paprika oleoresin ingested as a single dose (1 g) was not detected in plasma at all. However, the authors did not use a control group to study the bioavailability of the corresponding non-esterified xanthophylls but concluded that lipases did not cleave esters of oxocarotenoids such as capsanthin. Consequently, we designed a cross-over study using native 3R,3′R-zeaxanthin dipalmitate from wolfberries or non-esterified 3R,3′R-zeaxanthin, obtained after alkaline hydrolysis. The supplements were applied as one single dose together with a balanced breakfast. For the identification of zeaxanthin stereoisomers, a chiral HPLC column was used, and zeaxanthin enantiomers were confirmed employing chiral liquid chromatography (LC)–atmospheric pressure chemical ionisation (APcI)-MS. This allowed us to study whether or not *meso*-zeaxanthin appears in the bloodstream immediately after the ingestion of diets high in non-esterified or esterified 3R,3′R-zeaxanthin. To the best of our knowledge, this is the first report on chiral LC–APcI-MS of pooled human plasma samples.

Experimental design

Materials

Zeaxanthin stereoisomers (3R,3′R, 3R,3′S, 3S,3′S), 3R,3′R,6′R-lutein and γ-carotene were purchased from CaroteNature (Lupsingen, Switzerland). Acetone, 2,6-*di*-*tert*-butyl-4-methylphenol, diethyl ether, ethyl acetate, *n*-hexane, light petroleum (40–60°C), ethanol, and methanol were purchased from Merck (Darmstadt, Germany). Methyl *tert*-butyl ether and β-carotene were obtained from Fluka (Neu-Ulm, Germany). Test kits for the *in vitro* determination of plasma triacylglycerol (method TR 210) were purchased from Randox Laboratories GmbH (Krefeld, Germany); the kits for total cholesterol (method 401) were obtained from Sigma (Taufkirchen, Germany). All solvents were of analytical grade and were distilled before use. For HPLC, ultra-pure water obtained from a Milli-Q 185 apparatus (Millipore, Eschborn, Germany) was employed. Heat-dried wolfberries were kindly provided by RichNature Labs Inc. (19011 36th Avenue West, Suite G, Lynnwood, WA 98036, USA).

Subjects

Twelve human subjects were recruited from the staff and students of the Institute of Food Science (Hannover). The six females and six males were divided at random into two groups of six each. Three of the females used contraceptives. None of the individuals suffered from gastrointestinal diseases, or took laxatives or drugs lowering plasma triacylglycerol or cholesterol concentrations. Supplements containing carotenoids or vitamins were not consumed in the 2 weeks before the first blood sampling and during the study. The volunteers were requested to keep to a normal diet but to avoid zeaxanthin-rich food (for example, red and orange peppers, sea buckthorn, vitamin-fortified mixed fruit beverages) for the 2 weeks before and the 3 weeks during the intervention period. The study protocol was approved by the medical ethics committee of the Medizinische Hochschule, Hannover, and the participants provided written informed consent.

Study design

The study was designed as a single-blind cross-over study over a period of 23 d and consisted of two experimental days, separated by a 3-week depletion period during which no supplementation was carried out. At each of the intervention days, the subjects received a balanced breakfast with the respective supplementation outlined in Table 1. Carotenoid administration was performed by mixing a serving of 150 g commercial whole-milk yoghurt (3·5 % fat) with 10 g spiked sunflowerseed oil (see later; p. 709), respectively, resulting in a total amount of 5 mg 3R,3′R-zeaxanthin, independent of the non-esterified or esterified form. The yoghurt was consumed together with a standardised breakfast, described in an earlier study (Breithaupt *et al.* 2003). Due to the amount of oil suspension (10 g), the total fat content amounted to 29·9 g, the total energy to 2318 kJ. Blood samples were collected before (0 h) and after administration (3, 6, 9, 12, and 24 h). After 3 weeks, the cross over was performed (day 2).

Preparation of supplements

Extraction of carotenoids. Carotenoids were extracted from dried wolfberries using a ternary solvent mixture of

Table 1. Design of the single-blind cross-over study in which volunteers were divided at random into two groups and the two intervention days were separated by 3 weeks

Group	Day 1	Day 2
Group I (*n* 6)	3R,3′R-zeaxanthin dipalmitate	Non-esterified 3R,3′R-zeaxanthin
Group II (*n* 6)	Non-esterified 3R,3′R-zeaxanthin	3R,3′R-zeaxanthin dipalmitate

light petroleum–ethyl acetate–methanol (1:1:1, by vol.). Berries (500 g) were ground to a powder which was transferred into a flask and extracted (1 litre) by mixing thoroughly. The extract was separated from the plant tissue employing a Büchner funnel (VWR International Gmbh, Bruchsal, Germany). To allow for complete extraction, portions of solvent (200 ml each) were poured onto the filter cake and sucked off. This step was repeated until the filter cake was nearly colourless. The resulting extract was transferred into a separating funnel, washed with water containing a saturated solution of sodium chloride to allow for good phase separation. The water layer was discarded while the organic layer was transferred into an Erlenmeyer flask (VWR International Gmbh), dried with anhydrous sodium sulfate, filtered, and evaporated to dryness. The resulting residue was re-dissolved in light petroleum (400 ml) and stored at −20°C. For LC–APcI-MS analyses of native zeaxanthin esters, the residue of one additional preparation (20 g berries) was finally dissolved in *n*-hexane–iso-propanol (94·5:5·5 (v/v); 25 ml).

Saponification. A sample (200 ml) of the light petroleum solution (see above) was evaporated to dryness. The residual oil was dissolved in 500 ml diethyl ether and divided into five samples for simplified handling. For saponification, 5 ml methanolic KOH (30 %; w/v) was added to each portion and the mixture kept at room temperature for 12 h, protected from light. The ether phases were washed twice with water (100 ml each), dried with sodium sulfate, and filtered. The resulting residues were dissolved in light petroleum and combined (200 ml) and stored at −20°C. Since the extract contained predominantly 3R,3′R-zeaxanthin (>94 area %; see Fig. 1, chromatogram B, peak 3), it was not necessary to apply further clean-up steps.

Preparation of diets. For administration in the diet, the light petroleum was totally removed and the residues were dissolved in commercial sunflowerseed oil. The concentration of non-esterified 3R,3′R-zeaxanthin was adjusted to 0·512 (SD 9) μg/g (*n* 3), and the 3R,3′R-zeaxanthin dipalmitate concentration was set to 504 (SD 5) μg/g (*n* 3; measured as 3R,3′R-zeaxanthin after saponification) by dilution with oil. Using 10 g oil per diet, this resulted in an intake of 5 mg 3R,3′R-zeaxanthin in non-esterified or esterified form. Due to the low solubility of non-esterified 3R,3′R-zeaxanthin in oil, a stable emulsion was prepared whereas 3R,3′R-zeaxanthin dipalmitate formed a clear solution. Since both supplements were mixed with an excess of yoghurt (150 g), it was anticipated that there would be no effect on the resorption behaviour.

Verification of stability. The stability of 3R,3′R-zeaxanthin and 3R,3′R-zeaxanthin dipalmitate in sunflowerseed oil and in yoghurt was tested at the end of the study. After 3 weeks storing at −20°C, both spiked oils showed neither degradation nor isomerisation; likewise, carotenoid esters were stable in the yoghurt.

Plasma sample preparation

At any blood withdrawal, 5 ml were sampled from each participant. Immediately after collection, the plasma was separated from the erythrocytes by centrifugation (5000 rpm, 10 min) and stored at −20°C in plastic caps. The extraction of carotenoids from plasma was based on a method described by Khachik *et al.* (1997) and was described by Breithaupt *et al.* (2003). Extracts obtained were finally dissolved in 1 ml *n*-hexane–iso-propanol (94·5:5·5; v/v), passed through a 0·45 μm membrane filter and subjected to HPLC analysis. To determine possible losses during preparation, the HPLC peak areas of an internal standard (1 ml γ-carotene solution; 1 mg/l, dissolved in *n*-hexane) were monitored. The recovery from extractions of various samples was over 95 %. Plasma triacylglycerol and total cholesterol were measured by using commercial *in vitro* enzymic test kits.

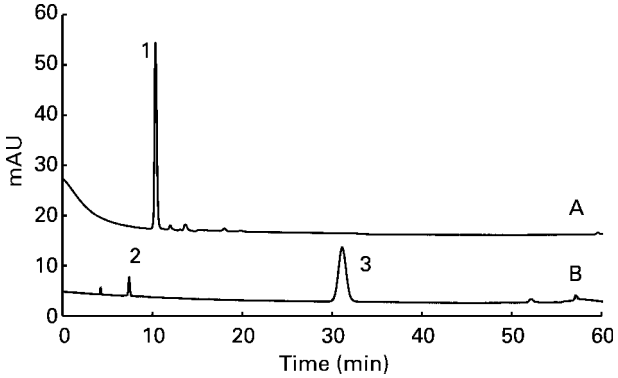

Fig. 1. HPLC chromatograms (extended sections; diode array detector, 450 nm) of diluted oil supplements containing native 3R,3′R-zeaxanthin dipalmitate (peak 1) from wolfberry (*Lycium barbarum*; chromatogram A) or non-esterified 3R,3′R-zeaxanthin (peak 3) obtained after chemical hydrolysis (chromatogram B). (2), Peak corresponding to β-cryptoxanthin. Peak assignment was carried out using liquid chromatography–atmospheric pressure chemical ionisation-MS analysis.

Analysis and chromatography

Carotenoids were quantified by HPLC on a Hewlett Packard (Waldbronn, Germany) HPLC Model 1100, using a diode array detector (450 nm) and a solvent gradient consisting of *n*-hexane (A) and iso-propanol (B) (Khachik *et al.* 2002). The column used was a ChiralPak AD (Chiral Technologies Europe, Bd. Gonthier d'Andernach, 67404 Illkirch, France), packed with silica gel (10 μm), coated with amylose tris-(3,5-dimethylphenylcarbamate) as selector. The following gradient (flow rate 0·7 ml/min) was used (min/% A): 0/94·5; 40/94·5; 50/85; 55/50; 90/50; 91/94·5; 120/94·5. LC–APcI-MS was run on a Micromass (Manchester, UK) VG platform II quadrupole mass spectrometer equipped with an APcI interface, operating in the positive mode (Breithaupt & Schwack, 2000). For confirmation of zeaxanthin enantiomers in human plasma, six plasma samples (group I, day 1) were pooled, evaporated to dryness, re-dissolved in 200 μl *n*-hexane–iso-propanol (94·5:5·5; v/v), and analysed with LC–APcI-MS.

Statistics

Results are expressed as mean values and standard deviations. Plasma concentrations were corrected by the

individual fasting 3R,3′R-zeaxanthin concentrations. Areas under the curve (AUC; 0–24 h) were determined automatically by the trapezoidal rule after the subtraction of fasting plasma 3R,3′R-zeaxanthin concentrations. For comparison of the plasma concentrations and the AUC of the two groups and days, two-sided *F* and *t* tests were employed. *P* values $<0{\cdot}05$ were considered statistically significant. All statistics were performed with Microsoft Excel 2000.

Results

Determination of carotenoids was carried out using an HPLC system equipped with a chiral phase (Khachik *et al.* 2002). Thus, the separation of 3R,3′R,6′R-lutein and zeaxanthin stereoisomers was achieved without further derivatisation. Other plasma carotenoids (for example, β-carotene, lycopene) were separated as well but were not studied in detail. The comparatively long running time (125 min) was necessary to obtain a good separation of the zeaxanthin stereoisomers and to allow for complete cleaning of the column. Fig. 1 depicts two chromatograms illustrating the constitution of both oil supplements: chromatogram A corresponds to the native xanthophyll pattern of wolfberries. Besides the main component, 3R,3′R-zeaxanthin dipalmitate (peak 1), only small amounts of other xanthophylls were present. Chromatogram B corresponds to a saponified sample. As anticipated, 3R,3′R-zeaxanthin (peak 3) formed the main component (>94 area %), accompanied by low amounts of non-esterified β-cryptoxanthin (peak 2). The enlargement of the peak width of 3R,3′R-zeaxanthin (peak 3) in trace B is due to the isocratic method used for chromatographic separation. However, equal peak areas proved that the concentrations were identical. As the concentrations of carotenoids other than 3R,3′R-zeaxanthin dipalmitate are very low, they probably will not affect the absorption behaviour of 3R,3′R-zeaxanthin dipalmitate and 3R,3′R-zeaxanthin. Co-chromatography with authentic 3R,3′R-zeaxanthin reference material proved zeaxanthin to be present only in the 3R,3′R-form. Since saponification with methanolic KOH does not influence stereochemistry, it was concluded that native 3R,3′R-zeaxanthin dipalmitate (peak 1) of wolfberries had the same enantiomeric profile. This observation is in accordance with the results of Maoka *et al.* (1986) who found that zeaxanthin in higher plants predominantly possesses the 3R,3R′ configuration (>99 %). Consequently, the diets administered to the volunteers contained 3R,3R′-zeaxanthin or 3R,3R′-zeaxanthin palmitate, respectively.

All participants were instructed to consume a low-carotenoid diet 2 weeks before and during the study in order to minimise the plasma zeaxanthin level. Fasting baseline concentrations of 3R,3′R-zeaxanthin averaged therefore only 0·095 (SD 0·009, range 0·08–0·12) μmol/l (*n* 12). Further characteristics of the participants (fasting plasma concentrations of cholesterol and triacylglycerol) on both intervention days are given in Table 2.

Confirmation of carotenoids in human plasma by LC–APcI-MS has been described earlier by Khachik *et al.* (1992) who used a nitrile-bonded column for separation. To the best of our knowledge, chiral LC–APcI-MS has not been used to confirm zeaxanthin stereoisomers in human plasma. Peak assignment was carried out in the positive ionisation mode using a chiral phase with amylose tris-(3,5-dimethylphenylcarbamate) as selector (Khachik *et al.* 2002). For unequivocal identification, characteristic ions (zeaxanthin *m/z* 569 $[M + H]^+$; lutein *m/z* 551 $[M + H\text{-}H_2O]^+$) and UV/Vis spectra (zeaxanthin 422/448/472 nm; lutein 418/442/470 nm) were used. Fig. 2 shows the chromatographic separation (extended section) of a standard mixture (line A) containing zeaxanthin enantiomers (3R,3′R (peak 3); 3R,3′S (*meso*) (peak 4); 3S,3′S (peak 5)) and lutein (peak 6) by LC–APcI-MS and the respective mass traces used for identification (line B, lutein *m/z* 551 $[M + H\text{-}H_2O]^+$; line C, zeaxanthin enantiomers *m/z* 569 $[M + H]^+$). Since lutein possesses one α-ionone end ring, water is easily lost from the quasimolecular ion, resulting in the formation of a predominant fragment ion at *m/z* 551. The zeaxanthin stereoisomers (line C) showed the anticipated quasimolecular ions (*m/z* 569). Lines D, E, and F correspond to the separation of a pooled plasma sample, obtained 24 h after the ingestion of 3R,3′R-zeaxanthin dipalmitate from wolfberries. The absence of mass signals at the typical retention times of *meso*- (peak 4) and 3S,3′S-zeaxanthin (peak 5) unambiguously proved the absence of both enantiomers in the plasma (lines E and F). Thus, besides other carotenoids as 3R,3′R,6′R-lutein (peak 6) and lycopene (peak 7), only the 3R,3′R isomer of zeaxanthin (peak 3) was detected. The same results were found in pooled samples of other

Table 2. Characteristics of the participants
(Mean values and standard deviations)

			Day 1		Day 2	
	Mean	SD	Mean	SD	Mean	SD
Age (years)*	26·8	5·4				
BMI (kg/m^2)*	23·1	2·9				
Fasting plasma concentrations†						
Total cholesterol (mmol/l)			4·17	0·55	4·13	0·74
Triacylglycerol (mmol/l)			1·25	0·61	1·13	0·45
3R,3′R-zeaxanthin (μmol/l)			0·09	0·01	0·10	0·01

* *n* 12 (six males and six females).
† *n* 12 (days 1 and 2 separate).

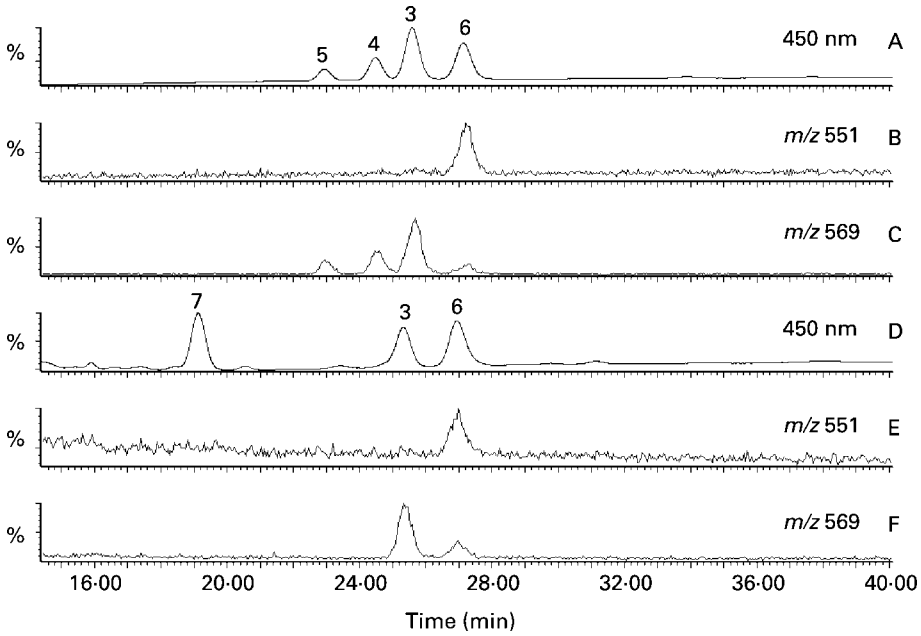

Fig. 2. Liquid chromatography (LC)–atmospheric pressure chemical ionisation (APcI)-MS analysis (extended sections) of a standard mixture (trace A) of zeaxanthin stereoisomers (3R,3′R (peak 3), 3R,3′S (peak 4) and 3S,3′S (peak 5)) and 3R,3′R,6′R-lutein (peak 6), and mass traces for lutein (*m/z* 551) (trace B) and zeaxanthin (*m/z* 569) (trace C). Trace D corresponds to the LC–APcI-MS chromatogram (diode array detector, 450 nm) of a pooled plasma sample after ingestion of 3R,3′R-zeaxanthin palmitate (3R,3′R-zeaxanthin (peak 3), 3R,3′R,6′R-lutein (peak 6), and lycopene (peak 7) and the corresponding mass traces for lutein (*m/z* 551) (trace E) and zeaxanthin (*m/z* 569) (trace F).

groups. Fig. 2 demonstrates that UV/Vis detection at 450 nm using a diode array detector even provides enough sensitivity for the analysis of pooled plasma samples. As potential *cis* isomers of lutein and zeaxanthin elute considerably after their corresponding all-*trans* forms (Khachik *et al.* 2002), co-elutions can be excluded. Additional LC–APcI-MS analyses gave no evidence for the presence of zeaxanthin esters. This is in accordance with the results of previous studies conducted with esterified lutein or β-cryptoxanthin as dietary supplements (Bowen *et al.* 2002; Breithaupt *et al.* 2003).

To determine the course of 3R,3′R-zeaxanthin increase, the average concentrations of plasma 3R,3′R-zeaxanthin of all the subjects were plotted *v.* the sampling time (Fig. 3). No matter whether the diets were supplemented with non-esterified or esterified 3R,3′R-zeaxanthin, the plasma concentration increased significantly ($P<0·05$), showing a maximum between 9 and 24 h in each case. The maximal plasma concentrations reached after supplementing non-esterified 3R,3′R-zeaxanthin were lower with smaller standard deviations (day 1, 0·044 (SD 0·008–0·013) μmol/l), compared with the supplementation with 3R,3′R-zeaxanthin palmitate, where higher concentrations and higher standard deviations were observed (day 1, 0·105 (SD 0·006–0·028) μmol/l). On the second intervention day the same situation appeared. Due to the high standard deviations observed after the ingestion of 3R,3′R-zeaxanthin dipalmitate, the concentration curves were statistically not distinguishable according to two-sided *F* and *t* tests ($P>0·05$). In order to account for potential variations in the total triacylglycerol (and cholesterol) concentrations, the individual values were corrected for both triacylglycerol and cholesterol. Each individual 3R,3′R-zeaxanthin concentration was divided by the

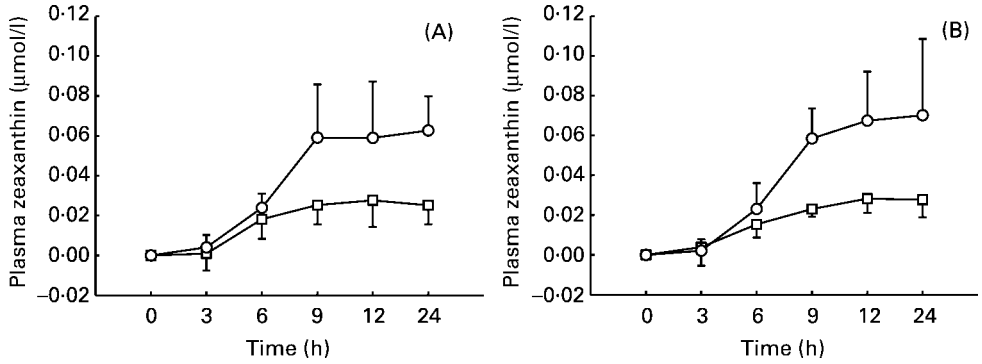

Fig. 3. Plasma 3R,3′R-zeaxanthin concentrations on day 1 (A) and day 2 (B) dependent on sampling time after ingestion of a single dose of non-esterified or esterified 3R,3′R-zeaxanthin (5 mg). Values are means, with standard deviations represented by vertical bars. (□), Non-esterified 3R,3′R-zeaxanthin; (○), 3R,3′R-zeaxanthin palmitate.

respective volunteer's mean triacylglycerol (or cholesterol) concentration and multiplied by the mean triacylglycerol (or cholesterol) concentration of all participants as a reference point. 3R,3′R-zeaxanthin plasma responses adjusted for triacylglycerol are shown in Fig. 4. Standardisation for total cholesterol revealed the same results (data not shown), proving that the results were not influenced by the individual serum lipid profiles of the human subjects.

To characterise the absorption behaviour of non-esterified and esterified 3R,3′R-zeaxanthin, a summary value implicating all sampled points, not only the maxima, is required. As suggested by Yao *et al.* (2000), the AUC was generated from the respective diagrams. This allows for an estimation of how effectively either of the two forms is resorbed. A two-sided *F* test ($P<0{\cdot}05$) revealed that the AUC for supplementing non-esterified 3R,3′R-zeaxanthin on days 1 and 2 had unequal variances; hence the corresponding *t* test ($P<0{\cdot}05$) had to be employed. Consequently, volunteers receiving the same diets on different intervention days were not pooled. The mean AUC (μmol/l × h) calculated that way for each day and group were as follows: non-esterified zeaxanthin (day 1), 0·492 (SD 0·18); zeaxanthin palmitate (day 1), 1·080 (SD 0·25); non-esterified zeaxanthin (day 2), 0·506 (SD 0·003); zeaxanthin palmitate (day 2), 1·178 (SD 0·18). Supplementation with 3R,3′R-zeaxanthin palmitate resulted in significantly higher AUC on both intervention days ($P<0{\cdot}05$); thus, AUC were distinguishable. Standardisation of the AUC to total triacylglycerol or cholesterol did not change the results. Therefore, serum triacylglycerol (or cholesterol) concentrations of the subjects had no influence on the results.

Discussion

Fasting baseline concentrations of zeaxanthin given in the literature range between 35 and 260 nmol/l (35 nmol/l (Khachik *et al.* 1992); 66 nmol/l (Grobusch-Klipstein *et al.* 2000); 260 nmol/l (Böhm & Bitsch, 1999)). The fasting baseline 3R,3′R-zeaxanthin concentrations determined were well within this range (81–116 nmol/l). Both total triacylglycerol and cholesterol concentrations were within the normal range for all the subjects (desirable concentrations of triacylglycerol and cholesterol are $<1{\cdot}25$ and $<5{\cdot}18$ mmol/l respectively; Jordan *et al.* 1995). After consumption of the 3R,3′R-zeaxanthin-fortified diets, the concentration maxima in the plasma were reached between 9 and 24 h after administration, whereas the highest concentration was 122 nmol/l (69 μg/l). Based on the assumption that 4 % of the body weight is plasma (2·4 litres plasma/60 kg body weight; Barua, 1999), this corresponds to an absolute amount of 166 μg 3R,3′R-zeaxanthin, which in turn corresponds to a percentage of 3·3 % of the administered dose. This is in accordance with data given in the literature, stating a low efficiency of carotenoid absorption, reported to range from 5 to 50 % (Olson, 1994). Interestingly, the amount absorbed corresponded to that found in an earlier study with β-cryptoxanthin esters (Breithaupt *et al.* 2003), although the carotenoid concentration administered in the present study was nearly four times higher. This underlines the tendency in man to absorb xanthophylls relatively poorly. The majority might have been metabolised to apocarotenals, which were not studied, or excreted through the gastrointestinal tract. The only stereoisomer found in the plasma after the ingestion of 3R,3′R-zeaxanthin palmitate was 3R,3′R-zeaxanthin. Thus, metabolism to the 3S,3′S enantiomer or the *meso* form is to be excluded in the plasma, in particular during the time period studied (24 h).

On comparison of the mean plasma concentrations after supplementing with non-esterified and esterified 3R,3′R-zeaxanthin, a higher standard deviation in the case of the esterified diet was observed. This made the absorption curves not distinguishable on both intervention days. However, the tendency agrees with earlier results using non-esterified and esterified β-cryptoxanthin (Breithaupt *et al.* 2003), where the ingestion of xanthophyll esters resulted in higher standard deviations, too. A reason for this behaviour is as yet unknown, though a possible explanation could be different lipase activity of individual human subjects.

AUC, which are directly proportional to the bioavailability (Yao *et al.* 2000), were calculated for each day and group. As the variances of the volunteers receiving non-esterified 3R,3′R-zeaxanthin were unequal when comparing both intervention days, the respective groups could not be pooled, resulting in four mean AUC, two for each day. The AUC resulting after the ingestion of 3R,3′R-zeaxanthin palmitate exceeded those obtained after the consumption of non-esterified 3R,3′R-zeaxanthin roughly by

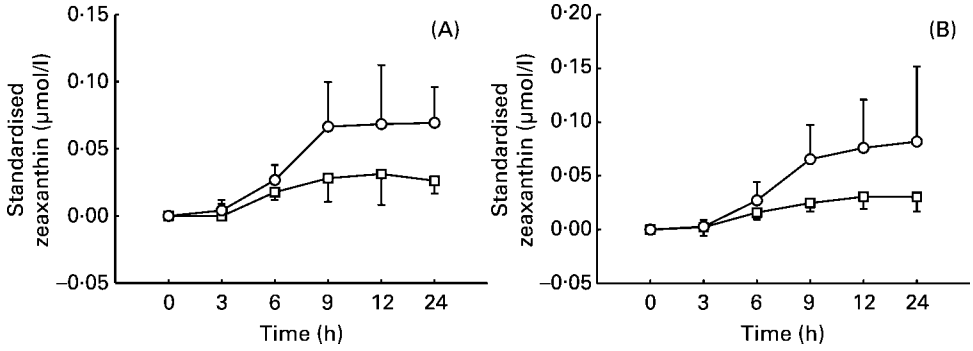

Fig. 4. Plasma 3R,3′R-zeaxanthin concentrations standardised to triacylglycerol plasma concentrations on day 1 (A) and day 2 (B). Values are means, with standard deviations represented by vertical bars. (□), Non-esterified 3R,3′R-zeaxanthin; (○), 3R,3′R-zeaxanthin palmitate.

a factor of two on both intervention days. The comparison of the AUC reveals that the low 3R,3′R-zeaxanthin concentration in plasma is not a result of low enzymic hydrolysation rates. If that were the case, it is anticipated that the AUC would be not distinguishable. The relatively low plasma 3R,3′R-zeaxanthin concentration is due, rather, to the low absorption tendency of the non-esterified form, no matter whether the xanthophyll is administered in its non-esterified or esterified form. In contrast to earlier studies (Bowen *et al.* 2002; Breithaupt *et al.* 2003), the plasma response after supplying esterified 3R,3′R-zeaxanthin was significantly higher than the response after the administration of the non-esterified form. The reason for an enhanced bioavailability of esterified 3R,3′R-zeaxanthin is not yet known. A possible explanation could be the apolar nature of 3R,3′R-zeaxanthin dipalmitate in comparison with the more polar non-esterified form, which allows for the effective formation of micelles, needed before lipase activity. A satisfactory explanation still requires further investigation.

Acknowledgements

We thank all volunteers of the Institute of Food Science, University of Hannover, Germany, who participated in the study. This work was supported by grant BR2173/1-1 of the Deutsche Forschungsgemeinschaft (DFG). We thank Professor Dr Wolfgang Schwack, University of Hohenheim, for the excellent working conditions at the Institute of Food Chemistry.

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