

Bioavailability of β -carotene (β C) from purple carrots is the same as typical orange carrots while high- β C carrots increase β C stores in Mongolian gerbils (*Meriones unguiculatus*)

Mandy Porter Dosti¹, Jordan P. Mills¹, Philipp W. Simon² and Sherry A. Tanumihardjo^{1*}

¹Department of Nutritional Sciences, University of Wisconsin-Madison, Madison, WI 53706, USA

²United States Department of Agriculture Agricultural Research Service, Vegetable Crops Research Unit, Department of Horticulture, University of Wisconsin-Madison, Madison, WI 53706, USA

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Vitamin A (VA) deficiency is a worldwide public health problem. Biofortifying existing sources of β -carotene (β C) and increasing dietary β C could help combat the issue. Two studies were performed to investigate the relative β C bioavailability of a β C supplement to purple, high- β C orange, and typical orange carrots using Mongolian gerbils (*Meriones unguiculatus*). In study 1, which used a traditional bioavailability design, gerbils (n 32) received a diet containing orange, purple, or white carrot powder, or white carrot powder + a β C supplement. In study 2, which included β C-biofortified carrots, gerbils (n 39) received orange, high- β C orange, purple, or white carrot powder in their diet. Both studies lasted 21 d and the gerbils were killed to determine the effect of carrot type or supplement on serum and liver β C, α -carotene, and VA concentrations. Liver stores of β C or VA in the gerbils did not differ between orange and purple carrot diets when equal amounts of β C from each of the diets were consumed ($P > 0.05$). Both the orange and purple carrot diet resulted in higher liver VA compared with the supplement ($P < 0.05$). High- β C carrots resulted in more than 2-fold higher β C and 1.1 times greater VA liver stores compared with typical orange carrots ($P < 0.05$). These results suggest that high- β C carrots may be an alternative source of VA to typical carrots in areas of VA deficiency. Second, phenolics including anthocyanins and phenolic acids in purple carrot do not interfere with the bioavailability of β C from purple carrots.

β -Carotene: Gerbils: Vitamin A: Carrots: Biofortification

Vitamin A (VA) deficiency is a worldwide public health problem in more than 118 countries; 100 to 140 million children are VA deficient. Of those VA-deficient children, up to 500 000 children will become blind every year; half of those children will die within 1 year of losing their sight (World Health Organization, 2003). Provitamin A carotenoids are a major source of dietary VA in a large proportion of the world's population and β -carotene (β C) is the most common provitamin A carotenoid (International Vitamin A Consultative Group, 1999). One long-term solution proposed to combat VA deficiency is gardening and preserving fruits and vegetables in order to maintain VA status (World Health Organization, 2003). Carrots (*Daucus carota* L.) are grown worldwide and a strain of carrots genetically selected to have high β C (high- β C orange) was developed in hopes of improving VA status in deficient areas of the world (Simon *et al.* 1989; Simon, 1990).

β C absorption from vegetables is highly variable and affected by a number of factors (Castenmiller & West, 1998; Tanumihardjo, 2002). Relative bioavailability of β C ranges between 19 and 34% in carrots (van het Hof *et al.* 2000), although this range is complicated by bioconversion to retinol. Furthermore, responses to orally ingested β C

appear to be somewhat independent of dose. Brown *et al.* (1989) found that 30 mg β C produced a peak response in plasma β C only 1.6 times higher than a 12 mg dose of β C, suggesting a higher β C intake decreases the efficiency of absorption. In addition, giving oral doses of either 60 or 210 mg of β C resulted in similar breast milk β C concentration in lactating women (Canfield *et al.* 1997).

Purple carrots have been reintroduced into modern production (Simon, 1997*a,b*) and may have potential health benefits due to their phenolic content. Epidemiological studies suggest that intake of fruits and beverages rich in polyphenols prevent disease. For example, red wine intake is associated with a decreased risk of CVD (Renaud & De Lorgeril, 1992; Criqui & Ringel, 1994). Purple carrots contain at least two classes of phenolic compounds; flavonoids and phenolic acids. One class of flavonoids, anthocyanins, has been described in purple carrots (Harborne, 1976, 1983, 1988; Glabgen *et al.* 1992*a,b*). Five different anthocyanins have been identified by HPLC–electrospray MS and tandem MS (Glabgen *et al.* 1992*a,b*); the major one identified as acetylated cyanidin glycoside, cyanidin-3-(2'-xylose-6'-feruloyl-glucose-galactoside) (Glabgen *et al.* 1992*b*). The major

anthocyanin identified was an acetylated cyanidin glycoside, cyanidin-3-(2'-xylose-6'-feruloyl-glucose-galactoside) (Glabgen *et al.* 1992a). The major phenolic acid is 5'-caffeoylquinic acid (Alasalvar *et al.* 2001).

Several studies show that the Mongolian gerbil (*Meriones unguiculatus*) is an appropriate model to investigate β C absorption, metabolism, and bioconversion to VA (Lee *et al.* 1999). Mongolian gerbils absorb β C intact at physiological doses and accumulate β C in liver, spleen, kidney, adrenal, adipose, and lung (Pollack *et al.* 1994). Mongolian gerbils convert β C to VA with similar efficiency to that of man (Lee *et al.* 1998) and β C consumption results in accumulation of hepatic and extrahepatic VA stores (House *et al.* 1997; Lee *et al.* 1998; Thatcher *et al.* 1998). Furthermore, once β C is removed from the diet, liver β C stores are rapidly depleted and not utilised for VA (Thatcher *et al.* 1998). No study to date has tested the effect of purple carrot phenolic compounds on β C absorption. Furthermore, it is not known if high- β C orange carrots will improve VA stores compared with typical orange carrots. The present study reports the relative bioavailability of β C from purple, typical orange and high- β C orange carrots as evaluated in two studies in Mongolian gerbils.

Experimental methods

Animals and diets

Male Mongolian gerbils (*Meriones unguiculatus*) were obtained from Charles River Laboratories (Kingston, NY, USA) at 35 and 41 d of age for studies 1 and 2, respectively. Post-weaning, animals were given a commercial rodent diet (Purina Mills, LLC, St Louis, MS, USA) by the breeder. Upon arrival, gerbils were individually housed in plastic shoebox cages and given free access to food and water. Gerbils were immediately placed on a purified VA- and carotenoid-free pelleted diet (Table 1) and acclimated to a powdered diet of the same composition (Table 1; study diet with white carrot powder), over 7 and 10 d for studies 1 and 2,

respectively. The purpose of the acclimation period was to allow gerbils to adjust to a powdered diet containing freeze-dried carrot powder and to stabilise VA stores. Room temperature and humidity were constant and a 12 h light–12 h dark cycle was maintained. Gerbils were weighed daily and monitored for health. After acclimation, gerbils were categorised by weight and then randomly assigned to treatment groups so that groups were weight matched. For both studies, gerbils were fed *ad libitum* diets containing carrot powder, and feed intake of each gerbil was determined daily. On day 21, gerbils were killed and liver and serum were collected to analyse concentrations of VA, β -, α -, and *cis*- β -carotene (most probably 9-*cis* in our analytical system). All animal handling procedures were approved by the University of Wisconsin-Madison Research Animal Resource Center.

Experimental design and diet preparation

Study 1 sought to compare the bioavailability of β C from anthocyanin purple and typical orange carrot powder to an equalised β C supplement. Study 2 sought to further investigate the bioavailability effects of carrot type between purple and orange and included a biofortified carrot type that had about twice the amount of β C per g carrot powder.

After the acclimation period in study 1, thirty-two gerbils received powdered diet (Table 1) containing white (two treatment groups), orange, or purple carrot powder (eight per treatment). Purple, orange, and white carrot powders were analysed for β C concentration (including *cis*- and *trans*- β C) before the start of the trial. Purple and orange carrot powders were added to the diets to have 50 nmol β C/g diet and white carrot powder was added to equalise carrot powder. White carrot powder was added to the white carrot diet to equal the total amount of carrot powder added to the other diets. The supplemented white carrot group was given β C dissolved in oil (two doses per d) that was equal to the amount of β C the purple carrot group received the previous day based on mean

Table 1. Composition of diets for acclimation period and for studies 1 and 2*

Ingredient	Pelleted diet (g/kg diet)	Carrot diet (g/kg diet)
Dextrose	463.5	458.1
Casein†	200.0	203.1
Sucrose	200.0	205.9
Cottonseed oil	40.0	40.7
Cellulose	50.0	44.2
Mineral mix‡	35.0	36.0
Vitamin mix§	5.0	5.2
L-Cystine	3.0	3.1
Carrot powder	–	28.4–33.0
Vitamin D ₃ (cholecalciferol; 500 000 IU/g) (mg)	39.7	40.9
Vitamin E (DL- α -tocopheryl acetate; 500 IU/g) (mg)	4.4	4.5
Choline dihydrogen citrate	3.5	3.6

* Except for the carrot powder, all dietary components were provided by Harlan Teklad, Madison, WI, USA.

† Vitamin free.

‡ AIN-93G-MX (Teklad, Madison, WI, USA).

§ Vitamin mix (Harlan Teklad, Madison, WI, USA) provided the following (mg/kg diet): biotin, 0.4; calcium pantothenate, 66.1; folic acid, 2; inositol, 110.1; menadione, 49.6; niacin, 99.1; *p*-aminobenzoic acid, 110.1; pyridoxine-HCl, 22; riboflavin, 22; thiamin-HCl, 22; vitamin B₁₂ (0.1% in mannitol), 29.7; ascorbic acid (97.5%), 1016.6. The vitamin mix did not contain any carotenoids or preformed vitamin A.

|| Freeze dried; total carrot powder was equal for all diets; white carrot powder was used to equalise the amount of carrot powder added for each diet. The amount of carrot powder added to each diet resulted in about 50 nmol all-*trans* β - + *cis*- β -carotene/g; 28.4 and 33.0 g carrot powder/kg for studies 1 and 2, respectively.

food intake and assuming the diet had 50 nmol β C/g diet. The two doses were given at least 4 h apart via displacement pipette into the gerbils' mouths. Purple and orange carrot diets were analysed for β C concentration one time each week during weeks 2 and 3 of the trial in duplicate. All gerbils were placed on a white carrot diet 4 h before kill to remove dietary sources of β C before kill to allow time for dietary β C to be absorbed. Based on an experiment by Pollack *et al.* (1994), the peak response of β C in serum after a test meal is at 4 h; therefore we assumed that β C in the diet would have been absorbed by 4 h. The supplement group was killed 1 d later and received the final supplement 4 h before kill.

After the acclimation period for study 2, thirty-nine gerbils received a powdered diet (Table 1) containing either white, high- β C orange, orange, or purple carrot powder (ten per treatment except white, nine animals). Purple and orange carrot powder was added to have 50 nmol β C/g diet and white carrot powder was added to equalise total carrot powder. Compared with study 1, the purple and orange carrot diets, as opposed to the carrot powder only, were analysed for β C in triplicate before and weekly during the trial. The purple and orange carrot diets were adjusted using the white carrot diet and reanalysed for β C in triplicate until equal concentrations of β C in the diets were achieved. Carrot powders were added to the high- β C orange and white carrot diets to equal the amount of powder in the purple and orange carrot diets. Concentration of β C in the high- β C orange carrot diet was analysed in triplicate during the last week of the trial.

Carrot powder and diet analysis

White (devoid of pigment) (Cg703-1), purple (Cg740-1), orange (Cg255-1), and high- β C orange (Cg712-5) carrots were grown in El Centro, CA, USA and Hancock, WI, USA. Carrots were peeled, sliced, and freeze dried in a Virtis specimen freeze dryer (The Virtis Company, Gardiner, NY, USA) at -10°C in the dark. Subsequent preparation of carrot powder, carrot diet, and analyses were done either in the dark or under yellow light. Carrot powder was finely ground using both a food processor and coffee grinder, homogenised with a mixer (Kitchenaid, St Joseph, MI, USA), and stored at -80°C until analysed and mixed into the diet. The diet was kept at -20°C during the trials. Carrot powder was analysed in triplicate for β -, α -, and *cis*- β -carotene before mixing into the diet. Carrot powder (10 mg) was placed into a 15 ml centrifuge tube and internal standard (β -apo-8'-carotenyl decanoate, 1 ml (6.2 nmol)), synthesised in the Tanumihardjo laboratory (Horvitz *et al.* 2004), was added and concentrated under Ar. Acetone-dichloromethane (10 ml, 50:50, v/v) was added to the sample and it was mixed by vortex and sonicated for 20 min in a sonicating water-bath. The sample was centrifuged at 1380 g for 1 min and the supernatant fraction decanted into a 50 ml volumetric flask. The sample was further extracted three times by addition of 10 ml acetone-dichloromethane (50:50, v/v), vortexed for 0.5 min, and centrifuged. The supernatant fractions were pooled and brought to 50 ml. A 1 ml sample was dried under Ar, reconstituted in 100 μ l methanol-dichloroethane (50:50, v/v), and 50 μ l was injected onto the HPLC system. White carrot powder (0.31 g) was analysed for carotenoids by the

method described earlier except the entire 50 ml extract was dried under vacuum using a rotary evaporator, re-dissolved in 100 μ l, and 75 μ l was injected onto the HPLC system. The method employed to analyse the carotenoids in freeze-dried carrot powder was tested against another published method (Deming *et al.* 2000) and yielded similar results when samples were not saponified.

Diets were analysed as described earlier for carrot powder with the following modifications: 0.8 g diet was used; internal standard was not concentrated under Ar before extraction; and 25 μ l of sample was injected on the HPLC system. CV for analysis of orange and purple carrot diets were 3.0 and 2.2% in study 1 and 8.4 and 8.1% for study 2, respectively. Mean CV for all carrot diets was 5.0 (SD 3.1)%. Employing the method described by Deming *et al.* (2000) to analyse diets containing freeze-dried carrot powder requires a saponification step that when applied to either diet or purified β C resulted in 50% or greater loss of β C from the sample in our hands. The method described earlier to extract β C from the diet resulted in similar values of β C as would be expected based on the amount of carrot powder added to the diet.

Carrot powder analysis for phenolic compounds

Purple and orange carrots were analysed by HPLC to identify classes of phenolic compounds. Carrots were also analysed by Folin-Ciocalteu (Singleton & Rossi, 1965) to determine total phenolic content. Carrot powder (2.1 g) was placed into a centrifuge tube and 20 ml acetone-water (70:30, v/v) was added to the powder. The mixture was sonicated in a sonicating water-bath for 20 min. The sample was centrifuged at 200 g for 15 min at 4°C and the supernatant fraction decanted. The extraction procedure was repeated two times, supernatant fractions pooled, and concentrated under vacuum evaporation at 40°C . The sample was reconstituted in 2 ml methanol-water (50:50, v/v) and a 50 μ l injection of a 1:20 dilution (in methanol-water, 50:50, v/v) was made onto a C18 column (Varian Dynamax, 60 A, 8 μ m, 4.5×250 mm; Varian Inc., Palo Alto, CA, USA) equipped with a guard column. The solvents for elution consisted of HPLC-pure water (solvent A) and methanol (solvent B); both solvents contained 13 mM-trifluoroacetic acid. Samples were run on a linear gradient system (2 ml/min) over 40 min from 100% A to 100% B and a 5 min reverse gradient to 100% A. The HPLC system consisted of a Waters automated gradient controller and two 501 HPLC pumps (Milford, MA, USA) and a Rheodyne 7125 manual injector (Rheodyne Inc., Cotati, CA, USA). Elution was monitored from 210 to 600 nm by a Waters 996 photodiode array detector and Millennium software (Milford, MA, USA) for collecting and analysing three-dimensional chromatograms.

Anthocyanins in the purple carrot were characterised by HPLC as described (Guisti & Wrolstad, 1996; Guisti *et al.* 1999a,b) with modifications and identified with purified standards (Hanabryggene Technology Center, Sandnes, Norway). Purple carrot powder (0.3 g) was placed in a 15 ml centrifuge tube and covered with Al foil during the extraction process to protect the sample from light. The sample was mixed with methanol (containing 2.65 M-formic acid (98% minimum)) by vortex, sonicated in a water-bath for 10 min, centrifuged at 1850 g for 10 min, and the supernatant fraction collected. Extraction was repeated until the pellet was colourless (five

extractions). Extracts were pooled and diluted to 50 ml. The sample (50 μ l of a 1:10 dilution in methanol–aqueous formic acid (2.65 M-formic acid in water), 90:10, v/v) was injected onto a Zorbax SB-C18 column (5 μ m, 4.6 \times 250 mm; Agilent Technologies Inc., Wilmington, DE, USA) maintained at 30°C (CH-30 column heater and TC-50 controller; Laboratory Systems Inc., Oconomowoc, WI, USA) and equipped with a guard column. The solvents for elution consisted of aqueous formic acid (solvent A) and methanol (solvent B). Samples were run (1 ml/min) using the following linear gradient program where the initial conditions were 95% A: (1) 20 min from 95% A to 45% A; (2) 0.5 min from 45% A to 0% A; (3) 5 min from 0% A to 95% A. The Waters HPLC system (Waters Corp., Milford, MA, USA) consisted of a pump control module, two 510 pumps, and a 712 automated injector. Elution was monitored from 250 to 600 nm with a 996 photodiode array detector and evaluated with Empower software. Chromatograms were generated at 530 and 280 nm to analyse anthocyanins.

Oil-dose preparation, administration and analysis

β C from softgel capsules (15 mg; General Nutrition Corporation Inc., Pittsburgh, PA, USA) was mixed into cottonseed oil (50 ml) and a minimum amount of acetone and dichloromethane was added to the oil to completely dissolve β C. Solvents were removed by a combination of evaporation under vacuum and blowing Ar through the oil. The concentration of β C in the oil dose was determined spectrophotometrically using an $E_{1\text{cm}}^{1\%} = 2592$ for β C (DeRitter & Purcell, 1981). For qualitative analysis of the carotenoid composition of the oil supplement, a sample of the oil dose was dissolved in dichloromethane–methanol (50:50, v/v) and analysed by HPLC.

Tissue extraction for carotenoids and retinoids

Tissues were kept in the dark or under yellow light. Blood was collected in BD Vacutainer™ Gel and Clot Activator SST by cardiac puncture while gerbils were under isofluorane anaesthesia (Abbot Laboratories, Chicago, IL, USA). Livers were removed and immediately placed on dry ice and subsequently transferred to -80°C until analysis. Blood was allowed to clot for 30 min at room temperature and centrifuged at 2700 g at 4°C for 10 min. Serum was placed in a cryovial, flushed with Ar, and stored at -80°C . For analysis, internal standard (β -apo-8'-carotenyl decanoate, 30 μ l (13.3 pmol)) was added to gerbil serum (0.30–1.0 ml) and mixed by vortex (0.5 min) with equal volumes of ethanol (0.1% butylated hydroxytoluene). The sample was extracted three times with the addition of hexanes (0.5–1.0 ml) and subsequent mixing by vortex (0.5 min) and centrifuging (1380 g; 0.5 min). Supernatant fractions were pooled and dried under Ar. The extract was reconstituted in 100 μ l dichloroethane–methanol (50:50, v/v) and 50 μ l analysed by HPLC. Mean extraction efficiency of the serum between the two studies was 82%. Livers were analysed for VA esters and retinol to determine total liver VA as described (Tanumihardjo *et al.* 1990) with modifications. Livers (0.4–0.8 g) were weighed and ground with anhydrous sodium sulfate in a mortar and pestle before adding an internal standard, all-trans- β -apo-8'-carotenol (0.6–1.0 ml (8.2–13.6 nmol), Fluka,

purified by HPLC). Internal standard was ground into the liver and the liver exhaustively ground and extracted several times with alternating dichloromethane and ethyl acetate to 50 ml. Extracts were filtered through no. 1 Whatman filter paper, dried under Ar (1–5 ml), reconstituted in 100 μ l methanol–dichloroethane (50:50, v/v), and finally 50 μ l was injected onto the HPLC system. Mean extraction efficiency of the livers between the two studies was 83%.

High-performance liquid chromatography analysis for carotenoids and retinoids

Carotenoids and retinoids from carrot powder, diet, and tissues were analysed by an HPLC system consisting of a guard column, Waters Resolve™ C18 column (5 μ m, 3.9 \times 300 mm), a Waters 600 solvent delivery system, 717 autosampler, and 996 photodiode array detector (Waters Inc., Milford, MA, USA). The mobile phase consisted of acetonitrile–water (95:5, v/v) (solvent A) and acetonitrile–methanol–dichloroethane (85:10:5, by vol.) (solvent B). Both solvents contained 10 mM-ammonium acetate and 7.2 mM-triethylamine. Samples were run at 2 ml/min using a gradient procedure: (1) 100% A for 3 min; (2) a 7 min linear gradient to 100% B; (3) a 15 min hold at 100% B; (4) a 2 min reverse gradient to 100% A. Absorbance was monitored from 210 to 550 nm and chromatograms were generated at 450 and 325 nm to quantify carotenoids and retinoids, respectively. Carotenoids and retinoids were identified by comparing their retention time and spectra with respective standards purified in our laboratory. Standard curves were generated with purified β C, α -carotene (α C), and retinyl acetate to determine tissue concentration of the carotenoids and VA.

Statistical analysis

Data were analysed using the general linear model procedure in the Statistical Analysis System (SAS OnlineDoc., version 8, Cary, NC, USA; SAS Institute Inc., 1999). Main effects were evaluated using one-way ANOVA. Differences among treatment groups were determined using a protected Fisher least-squares means test if the *F* test was significant. Differences were considered significant at $\alpha < 0.05$. For some analyses, data were log- or inverse-transformed before statistical analysis as indicated in footnotes of tables and figures in order to meet assumptions of normality for ANOVA. Contrasts were used to determine the difference in serum VA between orange, supplement, and purple *v.* white in study 1; and the difference between orange, high- β C orange, and purple *v.* white in study 2.

Results

Study 1

Diets and food intake. Carotenoid concentrations for the supplement and the purple and orange carrot diets were determined (Table 2). Even though the diets were initially mixed based on the β C concentration in the carrot powder, diet concentration of β C and α C was significantly higher in the purple carrot diet compared with the orange carrot diet ($P < 0.05$). White carrot powder contained 0.5 ng β C/mg freeze-dried

Table 2. Carotenoid concentrations of diets (nmol/g diet) (Mean values and standard deviations)

Study	Diet	n	βC		αC	
			Mean	SD	Mean	SD
1	Purple carrot	4	46.0 ^a	1.2 ^a	22.8 ^a	0.6
1	Orange carrot	4	40.4 ^b	1.2 ^b	15.1 ^b	0.4
1*	Supplement	2	49.9	1.6	ND	
2†	Purple carrot	12	48.4 ^a	3.9	25.3 ^a	2.0
2†	Orange carrot	12	47.9 ^a	4.0	19.3 ^b	1.5
2†	High-βC orange carrot	3	121.3 ^b	4.6	48.6 ^c	1.8

βC, β-carotene; αC, α-carotene; ND, not detectable.

^{a,b,c} Mean values within a column for each study with unlike superscript letters were significantly different ($P < 0.05$).

* Supplement values were not included in the analysis because of the differences in methods to determine concentration of the carotenoids.

† Samples were analysed in triplicate weekly before and during the study period except for the high-βC orange carrot diet.

carrot powder resulting in approximately 0.026 nmol/g diet. There was no detectable α- or *cis*-β-carotene in the white carrot powder. Analysis of the supplement by HPLC indicated that the carotenoids in the dose administered to the gerbils were 96.6% all-*trans*-βC and 3.4% *cis*-βC.

Total food intake, energy intake, and body-weight gain during the 21 d study were recorded and total βC intake was calculated (Table 3). Total food intake was the sum of the food consumed by the gerbils of each treatment group. Total βC intake was determined from the concentration of βC in the diet (Table 2) and total food intake (Table 3) except for the supplement group. βC intake for the supplement group was based on the concentration and amount of administered dose. Intake of βC was higher in the supplement group compared with the purple and orange diet groups ($P < 0.05$) and intake of βC was higher in the purple carrot group compared with the orange carrot group ($P < 0.05$). The purple carrot group had the highest food intake but was not different from that of the orange carrot group ($P < 0.05$) but was higher than that of the supplement and white carrot groups ($P < 0.05$). Food intake did not differ among orange carrot,

supplement, and white carrot groups ($P < 0.05$). The supplement group had the lowest body-weight gain compared with all other groups ($P < 0.05$); and weight gain for the white carrot group was lower than for the purple carrot group ($P < 0.05$) but was not different from that for the orange carrot group ($P < 0.05$).

Serum. Carrot diet type had a significant effect on serum VA and αC concentrations (Table 4). Diet did not affect serum concentrations of βC among purple carrot, orange carrot and supplement diets ($P > 0.05$); βC was not detected in the white carrot group. Serum αC was not detected in either the white carrot or supplement groups. The purple carrot diet resulted in the greatest increase in serum concentrations of αC compared with the orange carrot group ($P < 0.05$). The purple carrot group had the greatest serum concentration of VA, which was not different from the orange carrot group ($P < 0.05$) but was greater than the supplement and white carrot groups ($P < 0.05$). Serum VA did not differ between the orange carrot and supplement groups ($P < 0.05$); however, the orange carrot group had higher concentrations of serum VA compared with the white carrot

Table 3. Gerbil β-carotene (βC) intake, food intake, energy intake and weight gain during the 21 d study period (Mean values and standard deviations)

Study	Diet	n	Total intake of βC (nmol)*	Total food intake (g)		Energy intake (kJ)†	Body-weight gain (g)	
				Mean	SD		Mean	SD
1	Purple carrot	8	5639 ^a	122.6 ^a	6.5	1976	25.0 ^a	1.6
1	Orange carrot	8	4761 ^b	117.9 ^{a,b}	9.2	1900	24.0 ^{a,b}	3.4
1	Supplement	8	6117 ^c	109.9 ^b	10.8	1955	18.9 ^c	2.3
1	White carrot	8	2.9 ^d	112.7 ^b	5.8	1817	22.3 ^b	2.2
2	Purple carrot	10	5601 ^a	115.7	8.3	1865	19.5	3.4
2	Orange carrot	10	5429 ^a	113.3	8.9	1826	17.6	3.8
2	High-βC orange carrot	10	13264 ^b	109.4	7.5	1763	18.9	3.2
2	White carrot	9	3.4 ^c	110.3	8.4	1778	18.6	3.2

^{a,b,c,d} Mean values within a column for each study with unlike superscript letters were significantly different ($P < 0.05$).

* Total intake of βC was determined from the mean concentration of βC in the diet (see Table 2) and mean total intake of food over the 21 d study period except for the supplement group. Intake of the supplement group was determined by amount of administered supplement and its βC content. For study 2, total intake of βC data were log-transformed for statistical analysis.

† Energy intake of the supplement group included energy provided by the oil supplement. There were no significant differences in energy intake among groups in either study.

Table 4. Serum concentrations of vitamin A (VA) and carotenoids (α -carotene, α C and β -carotene, β C) (Mean values and standard deviations)

Study	Diet	n	VA (μ mol/l)*		β C (nmol/l)†		α C (nmol/l) †	
			Mean	SD	Mean	SD	Mean	SD
1	Purple carrot	8	2.11 ^a	0.43	34.8	25.2	18.6 ^a	10.7
1	Orange carrot	8	1.82 ^{a,b}	0.20	22.5	12.4	7.30 ^b	4.33
1	Supplement	7	1.72 ^{b,c}	0.16	22.2	25.2	ND	
1	White carrot	8	1.56 ^c	0.13	ND		ND	
2	Purple carrot	10	1.99 ^a	0.20	67.4	35.8	36.6 ^a	16.6
2	Orange carrot	10	1.71 ^{b,c}	0.17	47.3	12.1	20.1 ^b	4.88
2	High- β C orange carrot	10	1.86 ^{a,b}	0.15	101 ^a	43.9	44.1 ^a	19.9
2	White carrot	9	1.68 ^c	0.25	ND		ND	

ND, not detectable.

^{a,b,c} Mean values within a column for each study with unlike superscript letters were significantly different ($P < 0.05$).

* For study 1, data were inverse-transformed before statistical analysis. Serum VA is the addition of serum retinol and retinyl esters.

† Data were log-transformed before statistical analysis.

group ($P < 0.05$). Serum VA did not differ between the supplement and white carrot groups ($P < 0.05$). Using contrasts, serum VA among the purple carrot, orange carrot, and supplement group was compared with the white carrot group and was significantly higher ($P = 0.001$).

Liver. Neither β C nor α C was detected in livers from the white carrot group and α C was not detected in livers from the supplement group (Fig. 1). The purple carrot diet resulted in the largest increase in β C stores (Fig. 1 (A)) compared with the orange carrot and supplement groups ($P < 0.05$) and the largest increase in α C stores (Fig. 1 (C)) compared with livers from the orange carrot diet group ($P < 0.05$). Total β C stores did not differ between the orange carrot and supplement group ($P > 0.05$). The purple carrot diet resulted in the largest increase in VA liver stores (Fig. 1 (B)) compared with orange carrot, supplement, and white carrot diets ($P < 0.05$). Liver VA was higher in the orange carrot group compared with supplement and white carrot group ($P < 0.05$) and liver VA stores from the supplement group were higher than liver stores from the white carrot group ($P < 0.05$).

Study 2

Diets and food intake. Carotenoid concentrations for the purple, orange and high- β C orange carrot diets were determined (Table 2). The concentration of β C did not differ between the purple and orange carrot diets throughout the study period ($P > 0.05$). As expected, based on previous analysis of high- β C orange carrots, the β C concentration in the high- β C orange carrot diet was about two times higher than in the purple and orange carrot diets ($P < 0.05$). White carrot powder contained 0.5 ng β C/mg freeze-dried carrot powder, which results in 0.031 nmol β C/g diet in the white carrot diet. The high- β C orange carrot diet had a two times greater concentration of α C ($P < 0.05$) compared with the orange and purple carrot diets. Concentration of α C was greater in the purple carrot diet compared with the orange carrot diet ($P < 0.05$). Total gerbil food intake and body-weight gain were recorded and β C intake and energy intake were calculated, and they did not differ among treatment groups (Table 3). β C intake did not differ between the purple and orange carrot diets ($P > 0.05$) but intake of β C from the

high- β C orange carrot was 2.4 times greater than from the purple and orange carrot diets.

Serum. β C and α C were not detected in the serum of the white carrot group (Table 4). Serum from the high- β C orange carrot group had the greatest concentration of β C compared with orange and purple carrot diet treatments ($P < 0.05$). Serum α C concentration did not differ between purple and orange carrot treatments. Gerbils on high- β C orange carrots had the highest serum β C concentrations ($P < 0.05$) but were not different from the purple carrot diet; both purple and high- β C orange carrots resulted in higher serum α C concentrations than the orange carrot diet ($P < 0.05$). Gerbils receiving the purple carrots had the highest serum VA concentration, which was not different from the high- β C orange carrot, but was higher than the orange and white carrot treatments ($P < 0.05$). The high- β C orange carrot diet resulted in higher serum VA concentrations than the white carrot diet ($P < 0.05$), but was not greater than the orange carrot diet ($P > 0.05$). Serum VA from gerbils receiving either white or orange carrot treatments was not different ($P > 0.05$). Using contrasts, serum VA among the purple, orange, and high- β C orange carrot groups was higher than in the white carrot group ($P = 0.038$).

Liver. β C and α C were not detected in livers of the white carrot group (Fig. 2). The high- β C orange carrot treatment resulted in about two times greater liver stores of β C compared with the orange and purple carrot diets ($P < 0.05$; Fig. 2 (A)). β -Carotene liver stores did not differ between gerbils receiving the purple or orange carrot diets ($P > 0.05$). The high- β C orange carrot diet resulted in the largest increase in VA liver stores (Fig. 2 (B)) compared with the purple, orange and white carrot diets ($P < 0.05$). Liver VA stores did not differ between gerbils receiving the purple or orange carrot diet ($P > 0.05$), but liver VA from the purple and orange carrot diets was greater than in the white carrot group ($P < 0.05$). The high- β C orange carrot diet resulted in the greatest increase in α C liver stores (Fig. 2 (C)) compared with the orange and purple carrot diets ($P < 0.05$); the purple carrot diet resulted in higher liver α C compared with the orange carrot diet ($P < 0.05$).

Phenolic composition of carrot extract. Total phenolic content of purple and orange carrot was 18.5 and 6.6 mg

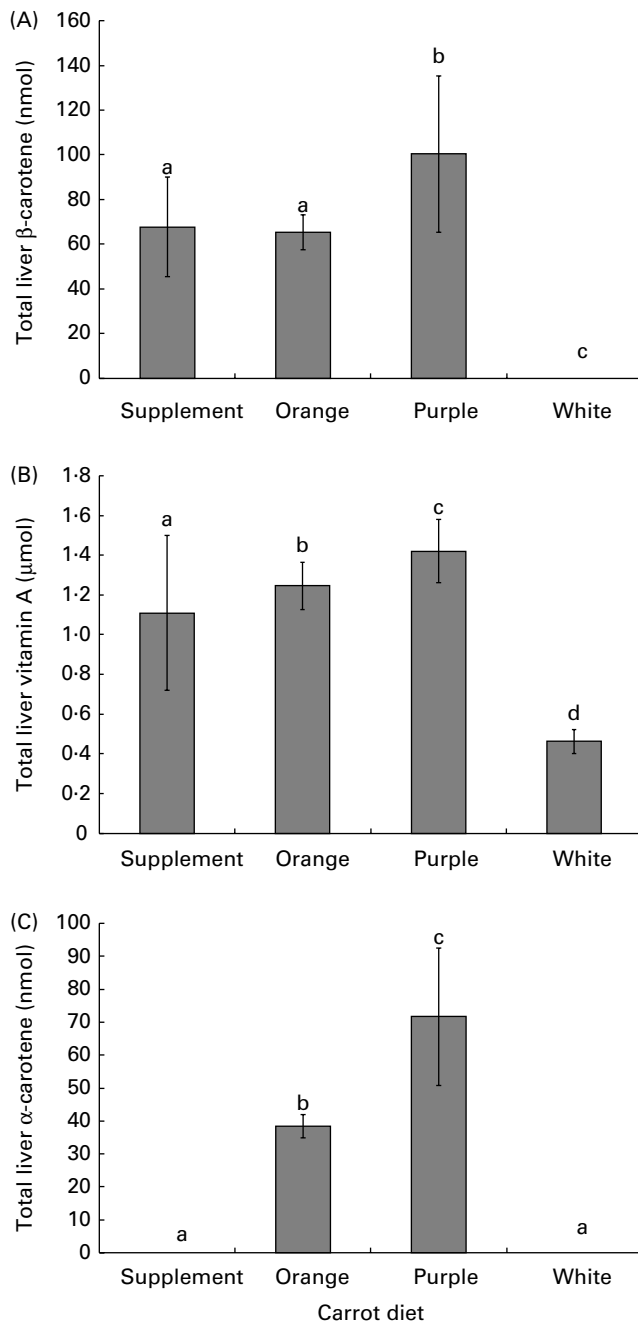


Fig. 1. Total hepatic stores of β -carotene (A), vitamin A (B) and α -carotene (C) from gerbils (*Meriones unguiculatus*) consuming a diet containing either orange, purple, or white carrot powder for 21 d (study 1). Gerbils in the supplement group also received the white carrot diet in addition to β -carotene administered in oil twice daily. Carotenoids were not detected in the white group livers. Data are the mean of each treatment (n 8), with vertical bars representing standard deviations. Data were log-transformed before analysis. ^{a,b,c,d} Mean values with unlike letters were significantly different ($P < 0.05$).

gallic acid equivalents/g dried carrot powder, respectively, as determined by Folin–Ciocalteu (Singleton & Rossi, 1965). Anthocyanins and cinnamic acids were the two main classes of phenolic compounds detected in the purple carrots and one class, cinnamic acids, was identified in the orange carrot. These results are consistent with other published results for purple and orange carrots (Alasalvar *et al.* 2001;

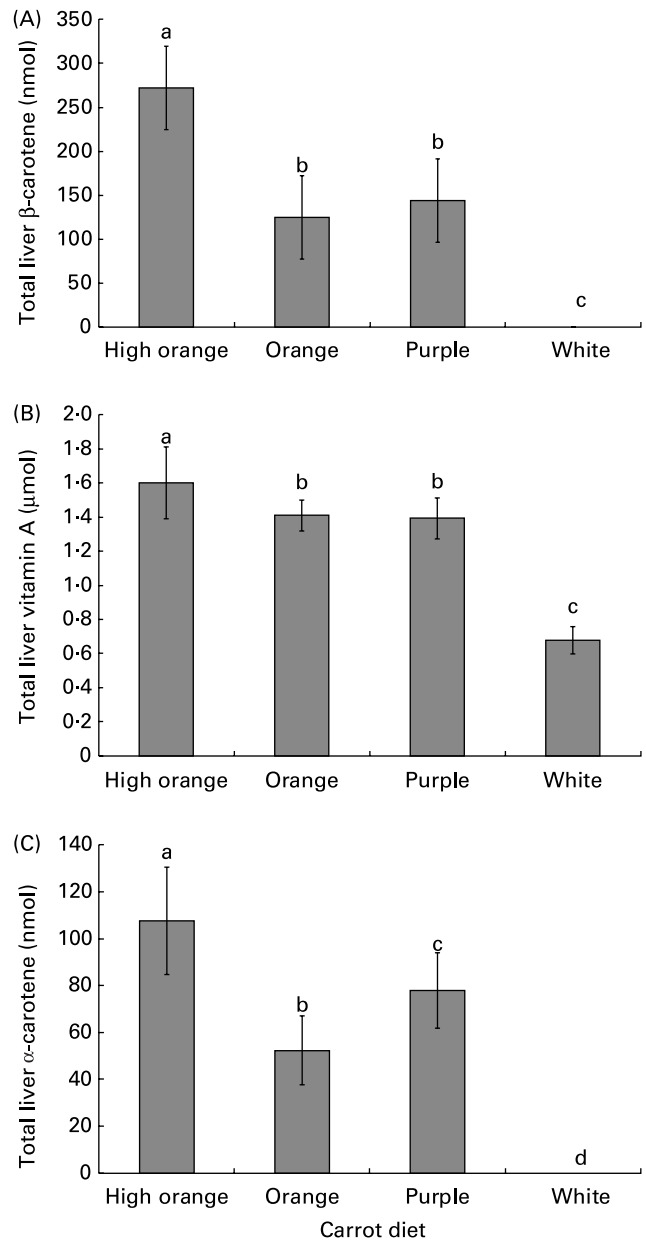


Fig. 2. Total hepatic stores of β -carotene (A), vitamin A (B) and α -carotene (C) from gerbils (*Meriones unguiculatus*) consuming a diet containing either orange, purple, high- β -carotene orange, or white carrot powder for 21 d (study 2). Carotenoids were not detected in livers from gerbils receiving the white carrot diet. Data are means (n 10, except for white group, n 9), with vertical bars representing standard deviations. Data were log-transformed before analysis. ^{a,b,c,d} Mean values with unlike letters were significantly different ($P < 0.05$).

Malien-Auburt *et al.* 2001). No anthocyanins were detected in orange carrots, whereas five of the following anthocyanins were identified in purple ones: cyanidin-3-(2'-xylose-6-glucose-galactoside), cyanidin-3-(2'-xylose-galactoside), cyanidin-3-(2'-xylose-6'-synapoyl-glucose-galactoside), cyanidin-3-(2'-xylose-6'-(4 coumuroyl) glucose-galactoside) and the major one, cyanidin-3-(2'-xylose-6'-feruloyl-glucose-galactoside). The anthocyanins detected in the purple carrot were the same as those described in the purple carrot root (Glabgen *et al.* 1992a). Evaluation of the spectra of peaks

showed that purple and orange carrot extract contained at least sixteen and six cinnamic acids, respectively (UV maxima of 330 nm) (Alasalvar *et al.* 2001). Cinnamic acids in the orange carrot were about 20% of the total cinnamic acids in the purple carrot.

Discussion

Studies looking at the interaction of different classes of dietary phytochemicals on nutrient status and bioavailability are few. These studies clearly show that phenolic compounds in purple carrots do not interfere with β C bioavailability and may even improve bioconversion as serum retinol concentrations were higher in both studies. Biofortification is one strategy hoped to improve VA status globally. Biofortification of carrots, a common food, resulted in higher β C and VA storage in an animal model with normal VA status.

In both studies, the relationship among treatments for total liver β C was the same as for total liver VA. Liver stores of β C were directly related to dietary intake of β C except for the supplement group. We attribute the differences in the effect of the purple carrot diet on β C liver stores between the two studies to unequal β C concentrations in the diet, as the first diet was mixed by carrot powder concentration and the second by diet concentration. Based on these two studies, we conclude that the phenolic compounds in purple carrots do not decrease the bioavailability of β C and may even enhance it. Liver stores of β C or VA did not differ between orange and purple carrot when diet concentrations are equalised. Interestingly, in both studies, the purple carrot diet tended to result in higher stores of β C. The β C intake was 1.18 and 1.03 times higher from purple carrot, in studies 1 and 2, respectively, compared with the orange carrot diet and liver β C stores were 1.54 and 1.15 times higher in gerbils consuming the purple compared with the orange carrot diet in studies 1 and 2, respectively. While there do not appear to be statistical differences in β C bioavailability between the purple and orange carrot diets, it is interesting that the purple carrot diet resulted in higher stores of liver β C.

β C from purple and orange carrots resulted in higher stores of VA compared with the supplement. Furthermore, β C from purple carrots resulted in higher β C in the liver despite lower β C intake than the supplement. Carrot powder was added into the orange and purple carrot diets to equal 50 nmol β C/g diet, the same as the amount of supplement given. The reason for the discrepant results between carrot and supplement could be that the gerbils were overloaded in β C dose and a large proportion of the dose bypassed absorption. The daily doses were given 4 h from one another, and each dose was between 100 and 150 μ l. In the analysis of a study we have conducted (Escaron & Tanumihardjo, 2006), most of the β C from a supplement in about 100 μ l oil is in the caecum by 3 h in Mongolian gerbils. Furthermore, because gerbils are predominantly grazers, the groups receiving β C from carrots received the β C throughout the whole day and therefore did not overwhelm their capacity to absorb the β C to the extent of the supplemented group. In contrast, plasma β C concentration of men chronically ingesting cooked carrots was 18% of the mean maximum change in plasma β C compared with those receiving supplements daily for 42 d (Micozzi *et al.* 1992). We freeze dried our carrots which may have

resulted in a more bioavailable source of β C compared with cooked or raw carrots. Also, men had a reduced ability to absorb higher doses of β C (Brown *et al.* 1989); perhaps our doses were too high for gerbils resulting in a decreased percentage of dose absorbed. The fact that the supplement group gained less weight than the other groups may have impacted the results. It is difficult to explain why the supplement group weighed less at the end of the experiment. We calculated that our oil supplement was about 10% of their total energy intake based on mean intake, which explains why the gerbils consumed less food. Energy intake was not different among treatment groups and, therefore, the decrease in body weight cannot be explained by differences in intake. The supplement group consistently gained less weight throughout the study period. While the supplement group body-weight gain was lower than in the other groups, their liver weights were not different (data not shown).

The purple carrots in the present study contained higher concentrations of α C compared with orange carrots. α C in the purple carrot diet was 1.5 and 1.3 times higher (Table 2), and resulted in 1.5 and 1.9 times higher α C liver stores compared with the orange carrot diet in studies 1 and 2, respectively. While few studies have investigated the effect of α C on β C absorption, Furusho *et al.* (2000) found that giving a mixture of α C and β C (1:2) decreased rat hepatic retinol stores compared with rats receiving β C alone. While the purple carrot diet contained higher amounts of α C, the α C does not contribute significantly to hepatic VA stores as there was no difference in VA stores between the orange and purple carrot treatments. However, considering that our gerbils were VA adequate, this result may be different in a more depleted VA state. In study 2, the high- β C orange carrot diet had 1.9 and 2.5 times the amount of α C compared with the purple and orange carrot diets, respectively, and hepatic α C was 1.4 and 2.1 times higher than with the purple and orange carrot diet treatments, respectively. These results provide inconclusive evidence of the effect of α C on hepatic VA stores during adequate VA status.

The high- β C orange carrot diet resulted in 1.9 and 2.2 times higher liver β C reserves compared with the purple and orange carrot diets, respectively, and dietary intake of β C was 2.3 and 2.4 times higher than in the purple and orange carrot groups. Furthermore, the high- β C orange carrot diet resulted in 1.1 times higher concentration of liver VA compared with the orange and purple groups. Therefore, despite the fact that the high- β C orange carrot diet contained twice the amount of β C, the liver VA stores were not twice as high. Furusho *et al.* (2000) found a similar result in that higher amounts of β C did not result in proportionally more VA stores in rats. Moreover, the white carrot group hepatic VA stores (242 nmol/g) were 3.5 times greater than the established cut-off for VA deficiency in experimental animals (70 nmol/g) (Olson, 1991), indicating that none of the groups were VA deficient but rather had adequate stores. Therefore, bioconversion of β C to VA may have been greater if VA stores were depleted. These results show that high- β C orange carrots are a better source of β C compared with 'typical' orange carrots because consuming the same amount of carrot resulted in 2-fold higher β C liver stores and 1.1 times higher VA liver stores. The application of these results to individuals in VA-deficient human populations needs to be further evaluated.

While serum concentrations are somewhat similar in ranking to liver carotenoid stores, if only serum concentration were used, the effect of carrot treatment on β C stores would be misinterpreted. For example, the purple and high- β C orange carrot groups had similar levels of β C in the serum, but the high- β C orange carrot group had twice the amount of β C liver stores than the purple carrot group. In addition, liver α C concentrations were higher in the high- β C orange carrot group compared with the purple carrot group but serum α C did not differ between the groups. Therefore the present results suggest that serum carotenoids are not sensitive indicators of carotenoid stores or bioavailability of carotenoids in gerbils. Other researchers have reported the absence of β C and α C in the serum of gerbils after feeding a diet containing those carotenoids for 3 weeks. The authors thought that the animals had been without feed for too long before the serum was sampled (Deming *et al.* 2000), whereas in human subjects, chronic dosing of carotenoids results in a constant elevated plateau of the carotenoids (Yeum & Russell, 2002). One explanation is that the lipoprotein profile of the Mongolian gerbil is different from that of man. In the Mongolian gerbil, the major circulating lipoprotein is HDL, not LDL. Mongolian gerbils have circulating lipoproteins composed of 62.5% HDL and 17% LDL (Nicolosi *et al.* 1981). In comparison, circulating lipoproteins in human subjects are about 30% HDL and about 50% LDL (Chapman, 1986). During fasting about 75% of carotenoids are associated with LDL, and the remaining with HDL and, to a lesser extent, VLDL (Erdman *et al.* 1993). Consequently, we believe that the Mongolian gerbil is not an appropriate model to test bioavailability of carotenoids by measuring serum carotenoid concentrations.

Serum VA reflected a similar pattern to liver VA in study 1 but not in study 2. The purple carrot diet resulted in the highest serum VA and was higher than the orange carrot diet whereas liver stores were the same. It is well known that serum VA concentrations do not reflect liver reserves of VA due to homeostatic control and recent dietary intake (Tanumihardjo, 2004). When both treatment and last-day food intake were included in a statistical model, both variables significantly affected serum VA in study 2; $P=0.0083$ and $P=0.025$, respectively. Serum retinol concentration was significantly higher in the purple carrot group perhaps due to the antioxidant status of those gerbils resulting in less catabolism or enhanced bioconversion. Studies to confirm this speculation, however, would be difficult to design in gerbils.

Carrots are a good source of β C and thus VA. While the increase in VA liver stores was modest in the present study with high- β C carrots, future studies in marginally deficient gerbils should be done as β C and α C were increased in a dose-dependent manner.

Substituting typical orange carrots with high- β C carrots could impact VA status in countries where carrots are consumed.

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