## Original Article

# Bioavailability and vitamin A value of carotenes from red palm oil assessed by an extrinsic isotope reference method

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Red palm oil (RPO) contains high concentrations of β- and α-carotene, and is presumed to possess a higher vitamin A value than other foods. The objective was to determine the metabolic vitamin A and carotene values of refined red palm oil in healthy adult subjects, using a stable isotope reference method. Twelve healthy subjects were administered a small standardised meal containing 10 g RPO (2.4 mg β-carotene and 1.8 mg α-carotene) in a blended juice-based drink also containing 2 mg tetradeuterated retinyl acetate ($d_4$-RA) as a metabolic reference. At baseline and at several times after the test meal, the concentrations of carotenes and of $d_4$- and $d_0$-(unlabelled) retinyl esters, in the plasma chylomicron-rich ($d < 1.006$) fraction were determined by high high-performance liquid chromatography and gas chromatography mass spectrometry, respectively. The masses of palm oil-derived vitamin A and carotenes absorbed ('yield') were calculated assuming 80% absorption of the $d_4$-RA reference dose. The mean yield of retinol from the RPO was 0.41 mg, ranging from 0.17 mg to 0.86 mg. The mean yields of β- and α-carotene were 0.29 mg and 0.25 mg, respectively, suggesting that β-carotene was more extensively metabolised than α-carotene. Subjects assimilated an average of 23% of the dose of carotenes, as the sum of retinol and unmetabolised carotenes. The vitamin A values of red palm oil obtained under these conditions, a mean of 0.17 mg retinol absorbed per mg β-carotene consumed (β-carotene : retinol equivalency of 5.7:1) is higher than that of all other vegetable sources we have evaluated to date.

**Key words: bioavailability, carotene, human, red palm oil, stable isotope, vitamin A.**

## Introduction

Vitamin A deficiency is one of the most prevalent and major nutritional problems in developing countries, especially in children of young age. In those nations, a substantial proportion of dietary vitamin A is commonly derived from β-carotene and other provitamin A carotenoids contained in fruits and vegetables. Therefore, it has long been encouraged to increase the consumption of carotenoid-containing fruits and vegetables to meet the vitamin A needs. However, the mass of vitamin A such commodities supply in actual meal situations is generally not known, owing primarily to a lack of methodologies to obtain such information.[1]

In contrast to the paucity of data on the vitamin A value of plant foods, there have been several studies concerning the relative ability of carotenoids from various sources to increase the plasma concentration of those carotenoids, the most common method of assessing carotenoid 'bioavailablity'.[2–5] These studies have revealed that carotenoid bioavailability is affected by many factors. Recently, van het Hof *et al.* have reviewed extensively the effect of dietary factors on the bioavailability of carotenoids.[6] Food matrix, fat and interactions between carotenoids are some factors that appear important. Carotenoids in plant foods appear to exist as either complexes with proteins or in crystalline form, suggesting that processing of vegetables by heat treatment and/or mechanical homogenisation (reduction in food particle size) may increase the bioavailability of carotenoids from such foods. Rock *et al.* reported an approximately three-fold increase in plasma β-carotene level following consumption of thermally processed carrots and spinach compared to raw vegetables, using two 4 week trial periods in a crossover design.[2] Castenmiller *et al.* demonstrated that disruption of the cellular matrix of spinach enhanced the bioavailability of β-carotene from whole leaf and minced spinach, but surprisingly without effect on lutein availability.[4]

Meal fat content appears to be another important factor in carotenoid bioavailability. Dietary fat (triglyceride) stimulates bile secretion from the gall bladder, which in turn facilitates the emulsification of carotenoids into mixed lipid micelles in the small intestine.[7] A complete absence of fat severely reduces β-carotene absorption.[8] More recent studies suggest that a modest amount of fat (3–5 g) appears to be sufficient to ensure intestinal uptake of carotene.[5,9]

Based on these findings it is reasonable to suggest that β-carotene in oil solution, without the digestive challenge provided by a solid food matrix, would promote maximal carotene absorption, with a correspondingly superior retinol

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equivalence. Indeed, the conventionally applied vitamin A mass equivalency of β-carotene in oil solution is lower than that of food-borne β-carotene.[10] Red palm oil (RPO) is one of the richest source of β- and α-carotene among all natural plant source of carotenes[11–13] and is now used as a cooking oil or ingredient in some regions. Several studies have demonstrated that RPO supplementation can improve vitamin A status in schoolchildren in India.[14–17] Manorama *et al.* observed a similar increase in serum vitamin A level following both RPO snack supplementation and oral vitamin A palmitate supplementation using the Modified Relative Dose Response (MRDR) assay, demonstrating that β-carotene from RPO was a good source of provitamin A for combating vitamin A deficiency.[14] Recently, van het Hof *et al.* compared the bioavailability of natural palm oil carotenoids with synthetic β-carotene by measuring the response of plasma carotenoids; both supplements increased plasma β-carotene by a similar extent with four days of supplementation.[18]

The objective of the current study was to directly determine the vitamin A and carotene equivalencies of refined RPO in healthy human subjects. To do so we applied a stable isotope reference method recently developed in our laboratory to estimate the masses of retinol and carotenes secreted into the bloodstream and associated with chylomicrons following a test meal containing provitamin A carotenoids.[19]

## Materials and methods

### *Subjects and study design*

All procedures were approved by the Cornell University Committee on Human Subjects. Exclusion criteria included smoking, hyperlipidaemias and liver malfunctions, as evidenced by preselection clinical screening. Subjects must not have used any vitamin supplement for 6 months prior to participation. Twelve healthy subjects (six women and six men) who met the criteria were enrolled. Subjects were instructed to avoid carotenoid-containing and vitamin A-rich foods for 24 h prior to their participation. Subjects fasted for the 12 h immediately preceding the trial.

A single-dose pharmcokinetic model was employed. After overnight fasting subjects were administered a single dose of 10 g red palm oil (Carotino Oil, Global Palm Products, Johor, Malaysia), incorporated into a blended juice drink. The 10 g RPO contained 2.37 mg β-carotene and 1.85 mg α-carotene, as determined by high-performance liquid chromatography (HPLC). The juice drink was prepared by first blending 60 mL diluted white pear juice (1:1, juice : water) and 30 g banana. One gram high oleate safflower oil (Spectrum Natural, Petaluma, CA, USA) containing 2 mg dissolved 10, 19, 19, 19-$H_2$ retinyl acetate ($d_4$-RA; Cambridge Isotopes, Cambridge, MA, USA) and 10 g RPO were slowly added to the blended drink while mixing. Subjects consumed the final blended drink with one plain bagel and then consumed 100 mL diluted pear juice. A serving of 100 mL water was used to rinse the drink container and palate to ensure complete ingestion of the dose. A small standardised lunch containing 5 g fat and an afternoon snack were provided at 3 and 6.5 h after the test meal, respectively. No other food was allowed throughout the study day.

### *Plasma collection and fractionation*

Blood samples were obtained in vacutainers containing $Na_2$-ethylenediamintetraacetic acid (EDTA; Sherwood Medical, St Louis, MO, USA) at baseline (prior to the test meal), and at 2, 3.5, 4.5, 5.5 and 8.5 h after the test meal. The samples were placed immediately on ice and centrifuged at $800 \times g$ for 10 min to obtain plasma. The triglyceride-rich lipoprotein (TRL) fraction ($d < 1.006$ g/mL) was obtained by a standard ultracentrifugation technique, as described previously.[19] Two different TRL preparations were constructed. For all 12 subjects, TRL fractions were prepared from the baseline plasma sample and from a post-dose plasma pooled sample, constructed using 3 mL aliquots of each of the five post-dose plasma samples. For six subjects, TRL fractions were prepared from each individual time point. To prepare the TRL fractions, triplicated 4 mL plasma samples were transferred to centrifuge tubes (13 × 64 mm, Bell-top Quick-Seal™ Centrifuge Tubes, Beckman Instruments, Palo Alto, CA, USA) and the tubes then filled to capacity with 2 mL of a $d = 1.006$ g/mL NaCl solution. The tubes were centrifuged at 100 000 g for 14 h at 4°C using a Beckman 50.3 Ti fixed-angle rotor and Beckman Model L5-50 ultracentrifuge. The top 1.5 cm of the tube ($d < 1.006$ g/mL fraction) was collected using a tube slicer; the slicer and tube were rinsed with 2 mL of a $d = 1.006$ g/mL NaCl solution and the rinse was combined with the $d < 1.006$ g/mL fraction. Samples were flushed with $N_2$ and stored at –80°C until analysis.

### *Extraction and analysis of vitamin A and carotenes in the TRL fraction*

Thawed samples were deproteinated with 3.5 mL absolute ethanol containing two internal standards: 0.1 μg echinenone (Fluka, Milwaukee, WI, USA) and 0.09 μg $d_8$-retinyl acetate (Cambridge Isotopes, Cambridge, USA). The sample was extracted twice with hexane (10.5 mL) and the combined extracts were dried under a stream of nitrogen gas. The residue was saponified with 1.6 mL 1% potassium hydroxide in ethanol at 60°C for 30 min, under nitrogen atmosphere in the dark, in order to convert the TRL retinyl esters to retinol and remove triacylglycerol. The sample was cooled, diluted with 1.2 mL distilled water and extracted twice with 2 mL hexanes. The hexane extracts were washed with 2 mL distilled water and divided into two equal portions. One of the portions was redissolved in 20 μL dimethylformamide and 130 μL methanol : acetonitrile (1:1, v/v) for HPLC quantification of carotenoids. The other portion was dried and silylated using 100 μL pyridine and 50 μL BSTFA (N,O-bis(trimethylsilyl)trifluoroacetimide; Pierce Chemical Company, Rockford, IL, USA) under nitrogen at 60°C for 30 min. The silylation reagents were evaporated under $N_2$ and the residue was redissolved in 30 μL heptane for retinol-TMS (trimethylsilane) quantification by gas chromatography mass spectrometry (GCMS).

The TRL concentrations of α-carotene and β-carotene were determined using the modified HPLC method of Thurman *et al.*[20] Echinenone was used as the internal standard as it was not detectable in native TRL fractions. The HPLC system consisted of a 15-cm Spherisorb ODS-2 column (Phase Separations, Norwalk, CT, USA), Waters 996 Photodiode Array Detector and 717 Plus Autosampler (Waters, Milford, MA, USA), Hewlett-Packard HP 1050 Series Isocratic Pump (Hewlett-Packard Company, Avondale, PA, USA) and Millenium Software Version 2.0 (Waters). The mobile phase consisted of methanol : acetonitrile : chloroform (47:47:6, v/v/v) with 0.05% triethylamine and 0.05 mol/L ammonium acetate, flow rate 1.0 mL/min. The retention times of echinenone, α-carotene and β-carotene were 8.5, 17.1 and 18.6 min, respectively. A β-carotene calibration curve using response values was used to quantify the plasma concentration of α- and β-carotene.

The trimethylsilyl derivatives of $d_0$-, $d_4$- and $d_8$-retinol were quantified by GCMS using a Hewlett-Packard 6890 gas chromatograph coupled to a Hewlett-Packard 5972 A mass selective detector. The gas chromatograph was equipped with a HP-1 methylsiloxane capillary column (30 m × 0.25 mm) operated in split injection mode with helium as the carrier gas and an injection volume of 1 μL. The oven temperature program was initiated at 220°C and ramped at 5°C/min to 280°C, then at 25°C/min to 300°C with an 8 min hold. In selected ion mode (SIM) the following ions were monitored (m/z): $d_0$-retinol, 358.5; $d_4$-retinol, 362.5; $d_8$-retinol (internal standard), 366.5. A retinol calibration curve using response values was used to quantify the plasma (TRL) concentration of $d_0$- and $d_4$-retinol.

### *Calculations*

For each analyte quantified (α-carotene, β-carotene and unlabelled ($d_0$) retinol, the latter derived from retinyl esters upon saponification), the baseline TRL concentration was substracted from the post-dose pool concentration (or individual time point concentration) to yield baseline-corrected concentrations, or 'response'. To estimate the mass of each analyte absorbed, we assumed an 80% of absorption efficiency of the $d_4$-RA extrinsic reference dose. Accordingly, the $d_4$-retinol-TMS response (baseline-corrected post-dose pool concentration) was set equivalent to 4.8 μmol, or 80% of the 6 μmol consumed. The response of each analyte was divided by that of the reference and multiplied by 4.8 μmol to yield the mass of that compound secreted into the bloodstream.

## Results

For all analytes, the maximum TRL concentration occurred between 2 and 5.5 h post-dose. The concentrations of all analytes had decreased and generally returned to near-baseline concentrations by 8.5 h post-dose, when the trials were terminated.

The mean masses of α-carotene, β-carotene and unlabelled vitamin A derived from the RPO test meal are given in Table 1. The mean yields of α- and β-carotene were 252 ± 133 and 292 ± 283 μg, respectively. The lower ratio of β-carotene to α-carotene in the TRL fraction relative to that in the RPO (0.67 compared with 1.28) suggests that β-carotene was more extensively metabolised to vitamin A than α-carotene. The mean yield of retinol was 406 ± 218 μg. The mass yield of each analyte was variable among subjects, but the greatest coefficient of variation was seen with β-carotene. There was a strong direct correlation ($r$ = 0.94) between the masses of α-carotene and absorbed β-carotene (Fig. 1). Among the 12 subjects, seven absorbed more retinol than β-carotene, reflecting efficient conversion of β-carotene to retinol in the intestinal mucosa. In the other six subjects, the mass of β-carotene absorbed intact was much higher than that of retinyl esters, reflecting a lower efficiency of conversion. Men tended to absorb more carotenes than women (Table 1), but the difference (21%) was not statistically significant.

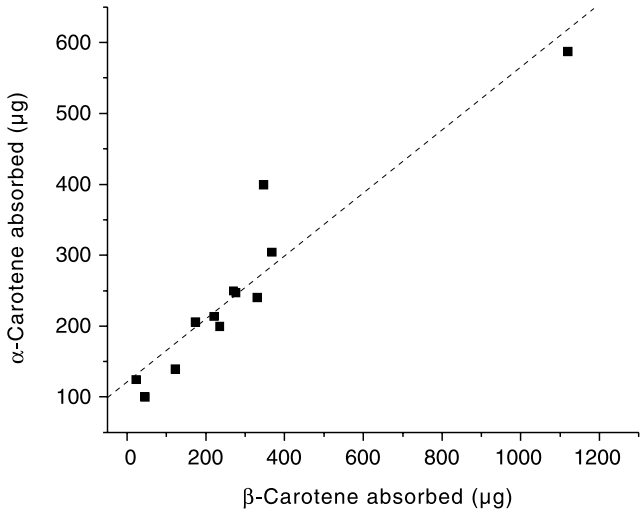


**Figure 1.** Relationship between the masses of absorbed β-carotene and α-carotene among 12 subjects consuming 10 g red palm oil. Each symbol represents a different subject, $r$ = 0.94.

**Table 1.** Estimated masses of retinol, α-carotene and β-carotene absorbed by 12 subjects consuming 10 g of red palm oil

| Sex | *n* | Mass of absorbed analyte (μg)† | | | | |
|---|---|---|---|---|---|---|
| | | αC | βC | ROH | αC + βC | αC + βC + ROH |
| Female | 6 | 228 ± 99 | 210 ± 118 | 402 ± 262 | 438 ± 212 | 839 ± 449 |
| Male | 6 | 276 ± 167 | 374 ± 382 | 409 ± 190 | 651 ± 547 | 1060 ± 537 |
| Total | 12 | 252 ± 133 | 292 ± 283 | 406 ± 218 | 544 ± 411 | 950 ± 486 |

†Values are given as mean ± SEM. αC, α-carotene; βC, β-carotene; ROH, retinol.

The proportion of ingested α- and β-carotene that was absorbed intact averaged 13.6% of the dose of 1.85 mg and 12.3% of the dose of 2.37 mg for α-carotene and β-carotene, respectively. Approximately 10% of the mass of total carotenes ingested was absorbed as retinol (retinyl esters). Overall carotenoid absorption, calculated as the total mass of carotenes plus retinol divided by the mass of carotenes consumed, averaged of 23% and ranged from 8 to 48%.

## Discussion

At present there is considerable interest and controversy regarding the relative merits of food-based vesus pharmacological approaches to improving the vitamin A status of populations in developing countries.[15,21] Much of the controversy stems from a paucity of quantitative data on the vitamin A equivalence of foods containing provitamin A carotenoids. Some recent studies have suggested that plant foods may not provide as much vitamin A as previously thought[22] and that some plant foods may possess greater vitamin A value than others.[23] It has been presumed that the lower vitamin A value of carotene-containing foods, relative to vitamin A itself or to purified β-carotene preparations, stems from poor release of carotenes from the food matrix during digestion. Red palm oil, in which the carotenes are dissolved in a highly digestible matrix, in principle provides a food ingredient of superior vitamin A value. We tested that hypothesis using an extrinsic stable isotope reference method developed recently in our laboratory.[24] This approach involves coadministration of a small amount of deuterium-labelled vitamin A ester (acetate), dissolved in oil solution, with a test meal containing the commodity of interest. One compares the concentration versus time response, in the plasma chylomicron-rich fraction, of the vitamin A and carotenes derived from the commodity with that of the labelled vitamin A reference dose. The mass of vitamin A and carotenes derived from the test commodity is then calculated, assuming that the labelled reference dose is absorbed with an efficiency of 80%. This approach has the distinct advantage of internally controlling for between-subject differences in the kinetics of chylomicron secretion and clearance, blood volume, and for recovery of carotenes and retinyl esters during sample preparation and analysis.

Using this method, we found that in our well-nourished subjects an average of 23% of the combined dose of 2.37 mg β-carotene and 1.85 mg α-carotene was secreted into the bloodstream, with an average retinol yield of 0.41 mg, or 0.17 mg retinol per mg β-carotene consumed. The observed β-carotene : vitamin A equivalency ratio of 5.7:1 (5.7 mg β-carotene yielding 1 mg retinol, assuming that none of the vitamin A was derived from α-carotene) for RPO is substantially higher than those determined for other plant foods using this same approach. For example, raw carrot and spinach, supplying 6 mg β-carotene, gave retinol equivalency ratios averaging approximately 23:1.[19] Using field-based interventions in vitamin A marginal populations, de Pee *et al.* have proposed equivalency ratios of 26:1 for vegetables and 12:1 for some fruits.[23] Earlier, an equivalency ratio of 2:1 was proposed for β-carotene in oil,[10] a value obtained using chronic feeding studies in vitamin A-depleted subjects, but which has not been replicated. The higher ratio (lower vitamin A value) observed for RPO in this study may be representative of well-nourished subjects, or reflect a single bolus dose rather than multiple smaller doses. In addition, this approach measures only that vitamin A which is formed in the intestinal mucosa during carotene absorption, and not that which may be formed post-absorptively, although the extent of the latter is unknown at present. Our data also show a trend for greater bioavailability of RPO carotenes in men than in women, although the observed difference of 21% was not statistically significant given the variation between subjects and the relatively small sample size.

Even with this solids-free oil matrix and a small, highly standardised test meal, there was a surprising degree of between-subject variation in the absolute bioavailability of RPO carotenes. The proportion of ingested carotenes absorbed, as the combination of carotenes plus retinyl ester, ranged from 8 to 48%. Because each subject was tested only once, it is not known whether those subjects exhibiting low or high absorption efficiencies were consistent in this regard. However, subjects who absorbed relatively higher amounts of β-carotene also absorbed greater amounts of α-carotene. Interestingly, the coefficient of variation in mass absorbed was substantially greater for β-carotene than for α-carotene or retinol. This suggests that there exist physiological factors, variable between individuals, that influence β-carotene uptake and secretion more than those of α-carotene or retinyl esters.

The lower β-carotene : α-carotene ratio in the chylomicron fraction relative to that in RPO suggests a greater proportion of absorbed β-carotene was converted to vitamin A, relative to α-carotene. This observation is consistent with our previous studies of carrots, which also contain both α- and β-carotene. However, again consistent with previous findings, there was no relationship, either direct or inverse, between the mass of absorbed β-carotene and the mass of absorbed vitamin A. This suggests that the amount of vitamin A formed via the metabolism of carotenes in the intestinal mucosa is influenced (regulated or otherwise limited) by factors other than the supply of substrate.

Our results support the concept that red palm oil is a food product possessing a vitamin A equivalency superior to that of solid foods. Red palm oil has been successfully used to improve vitamin A status,[16,17] consistent with studies employing experimental oil solutions of β-carotene,[22,25] and this study helps to explain the physiological reason behind this success in a more quantitative fashion. Continued and increased use of red palm oil as a dietary ingredient seems warranted, particularly in regions that continue to suffer from vitamin A malnutrition.

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