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Bioavailability of the isomer mixture of phytoene and phytofluene-rich alga *Dunaliella bardawil* in rat plasma and tissues

Moshe J. Werman[a], Shoshana Mokady[a], Ami Ben-Amotz[b,*]

[a]*Department of Food Engineering and Biotechnology, Technion, Haifa, Israel*
[b]*The National Institute of Oceanography, Israel Oceanographic and Limnological Research, Haifa, Israel*

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Abstract

Dunaliella bardawil, a β-carotene-accumulating alga was treated by the bleaching herbicide norflurazon to select sub-species rich with a mixture of 9-*cis* and *all-trans* stereoisomers of phytoene and phytofluene. The present study determines the bioavailability of phytoene and phytofluene with their stereoisomers in rats fed on a diet supplemented with *Dunaliella* phytoene-rich spray dried powder. Three groups of female weanling rats, eight animals each, were fed AIN diets for two weeks. The control consumed the diet as is. The experimental group was supplemented with 50 g *Dunaliella* powder to give phytoene/phytofluene at a level of 1 g/kg diet, and the placebo was provided with the oxidized algae free of carotenoids at the same amount. Weight gain and tissues weight of rats fed on the control diet, or on the experimental diets were statistically same. Tissue analyses were carried out by liquid chromatography at the end of two weeks feeding for vitamin A, carotenoids, phytoene and phytofluene and theirs stereoisomers. Liver analyses revealed high hepatic storage of phytoene in the experimental group. Analysis of the other tissues, adrenal, brain, heart, kidney, lung, and spleen detected small amounts of phytoene in the adrenal, kidney and spleen and in the plasma. High-pressure liquid chromatography for stereoisomeric composition was performed to all phytoene-containing tissues. The original algal diet content of 9-*cis*-to-*all-trans* ratio of 1:1 was maintained in the plasma and adrenal while in the liver, spleen and kidney the ratio was reduced to 1:3. The preferential accumulation of *all-trans* phytoene over 9-*cis* phytoene in the liver, spleen and kidney may be interpreted as indicating stronger antioxidative effect of 9-*cis* phytoene over the *all-trans* isomer or alternatively, *in vivo* streoisomerization of 9-*cis* phytoene to the *all-trans* structure. © 2002 Elsevier Science Inc. All rights reserved.

Keywords: Bioavailability; *Dunaliella*; Phytoene; Phytofluene

1. Introduction

Epidemiological and clinical studies suggest that a diet rich in fruits and green and yellow vegetables reduces the risk of cancer and other chronic diseases. It has been argued whether carotenoids are the biological active agents responsible for the reduction of cancer risk associated with fruits and vegetables consumption [1,2]. Since β-carotene is present in abundance in these vegetables and fruits and is available as synthetic commercial product it has been investigated extensively as the possible cancer preventive agent. However, large intervention studies which were published in the last decade noted carcinogenicity in high-risk population of heavy smokers and asbestos workers taking large doses of *all-trans* β-carotene [3–8]. The negative clinical effect of the synthetic *all-trans* β-carotene drew the attention to other natural carotenoids coexisting with β-carotene in vegetables and fruits as potent antioxidants in the prevention and reduction of certain chronic diseases.

The lipophilic biosynthetic pathway of carotenoids in plants originates from phytoene, the three double bonds colorless hydrocarbon, and thereafter forms phytofluene, five double bonds hydrocarbon, through a few intermediate colored carotenoids to β-carotene [9]. Significant amounts of phytoene are located in ripening tomatoes, but the abundance of phytoene in most other fruits and vegetables is small and its extraction is elaborated, thus, no studies were performed on the bioavailability of phytoene in mammals.

The unicellular alga *Dunaliella bardawil* has received much attention in recent years as the richest natural source of β-carotene [10]. The unique ability of the alga to accumulate large amounts of β-carotene has been investigated thoroughly [11–13]. The algal β-carotene is composed of mainly two stereoisomers *all-trans* and 9-*cis* in approxi-

* Corresponding author. Tel.: +972-4-8515202; fax: +972-3-5351378.

E-mail address: AMI.OCEAN.ORG.IL (A. Ben-Amotz).

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mately equal amounts. This stereoisomer mixture was shown to accumulate to a higher extent in livers of rats and chicks than synthetic *all-trans* β-carotene. The algal β-carotene also demonstrated high antioxidative properties in human studies under normal nutritional conditions [14], and was shown to cope more efficiently with dietary induced oxidative stress caused by feeding oxidized soybean oil or chronic alcohol consumption in rats, respectively [15–17].

Earlier reports showed that *Dunaliella bardawil* treated and inhibited at the phytoene desaturase by the bleaching herbicide norflurazon accumulates large quantity of the colorless carotenoids, phytoene and phytofluene each composed of equal isomeric ratio of *all-trans* and 9-*cis* [18–20]. The present study took advantage of the recent large-scale cultivation and biomass production of the phytoene/phytofluene-rich *Dunaliella bardawil* to determine the bioavailability of the algal phytoene stereoisomers in rat serum and tissues.

2. Materials and methods

2.1. Phytoene source

The natural phytoene source was provided as a spray-dried *Dunaliella bardawil* vacuum packed powder, a product of N.B.T. Ltd., Eilat, Israel. Selected phytoene-rich algae were cultivated outdoors in large-scale open raceways [12] and treated by bleaching herbicides as previously described [18–20]. The algae were harvested by centrifugation, washed for salt removal and spray dried to yield algal powder containing 2% phytoene, 0.5% β-carotene and 0.1% phytofluene. Analysis by 3-D photodiode array high performance liquid chromatography (HPLC), [18–20] supported by on line fluorescence showed that each of the algal carotenoids was composed of two major stereoisomers, *all-trans* and 9-*cis* in a ratio closed to 1:1. The phytoene-rich spray-dried *Dunaliella* was oxidized by breaking the vacuum and exposure the algal powder to light and air at 30°C for a few weeks monitoring kinetically the oxidation of the algal pigments. Oxidized spray-dried *Dunaliella* contained less than 0.05% carotenoids.

2.2. Animals and diets

Weanling Sprague-Dawley female rats (obtained from the animal colony of the Department of Food Engineering and Biotechnology, Technion, Haifa, Israel) were divided into three groups of eight rats each, having an average initial weight of 59.8 ± 6.0g. The animals were housed in wire cages in a room maintained at 23°C with light-dark cycle of 12 hr, and were fed for two weeks ad libitum a commercial AIN diet. The control group consumed the diet as is, whereas the two other groups were fed diet supplemented either with 50g of phytoene-rich *Dunaliella* to give 1g/kg phytoene -experimental, or the same amount of the oxidized algae placebo.

At the end of feeding period the animals were euthanized by carbon dioxide asphyxiation. Following perfusion with cold isotonic saline, the livers, and other tissues were removed, blotted and frozen at -70°C. Blood was collected over EDTA from the abdominal aorta and centrifuged at 1,000 x g for 25 min at 4°C, and plasma samples were stored at -70°C for further analysis.

2.3. Pigments and retinol analyses

Livers, spleens, kidneys, hearts, lungs and brains were assayed in wet state for vitamin A and carotenoids with saponification of vitamin A as previously described [21–24]. Total β-carotene stereoisomers, phytoene, phytofluene and retinol were determined colorimetrically at 475 nm ($E^{1\%}_{1cm}$ = 2,300), 287 nm ($E^{1\%}_{1cm}$ = 915), 348 nm ($E^{1\%}_{1cm}$= 1350), and 325 nm ($E^{1\%}_{1cm}$ = 1,600), respectively. The content of β-carotene and phytoene stereoisomers of the algal powder, the plasma and the tissue extracts was determined by HPLC as previously described [25]. Phytofluene was detected qualitatively by its typical fluorescence emission at 520 nm following excitation at 350 nm (Jasco Flourimeter model FP-1520, Ishikawa, Japan). The fluorimeter was connected in sequence to the HPLC photodiode array to provide matching elution chromatograms of both absorption and fluorescence with a short time difference of 15 s between the two. A stainless steel column of 25 cm x 4.6 mm (inner diameter) packed with C18 reversed phased material of 5 μm particle size, and a guard column (Vydac 201 TP 54, The Separation Group, Hesperia, CA USA) were used. Elution was performed with an isocratic solvent, methanol : acetonitrile (9:1, v/v) at a rate of 1 ml/min. The identification of the stereoisomers was done by absorption, fluorescence and elution time comparison to authentic standards obtained from Hoffmann La Roche, Basel, Switzerland.

2.4. Statistical analysis

Statistical studies were performed using SAS/Stat Version 6.04 software (SAS Institute, Cary NC USA). Data were analyzed by one-way analysis of variance followed by Duncan's multiple range test. A probability level of 0.05 was selected as the point at which differences were considered significant. Data are presented as means ± SD.

3. Results

Analysis by 3-D photodiode array in sequence with fluorimeter HPLC showed that the major pigments in the phytoene-rich powder of *Dunaliella bardawil* were *all-trans* phytoene and 9-*cis* phytoene in a ratio close to 1:1 (Figure 1). Phytofluene with its two stereoisomers was detected by

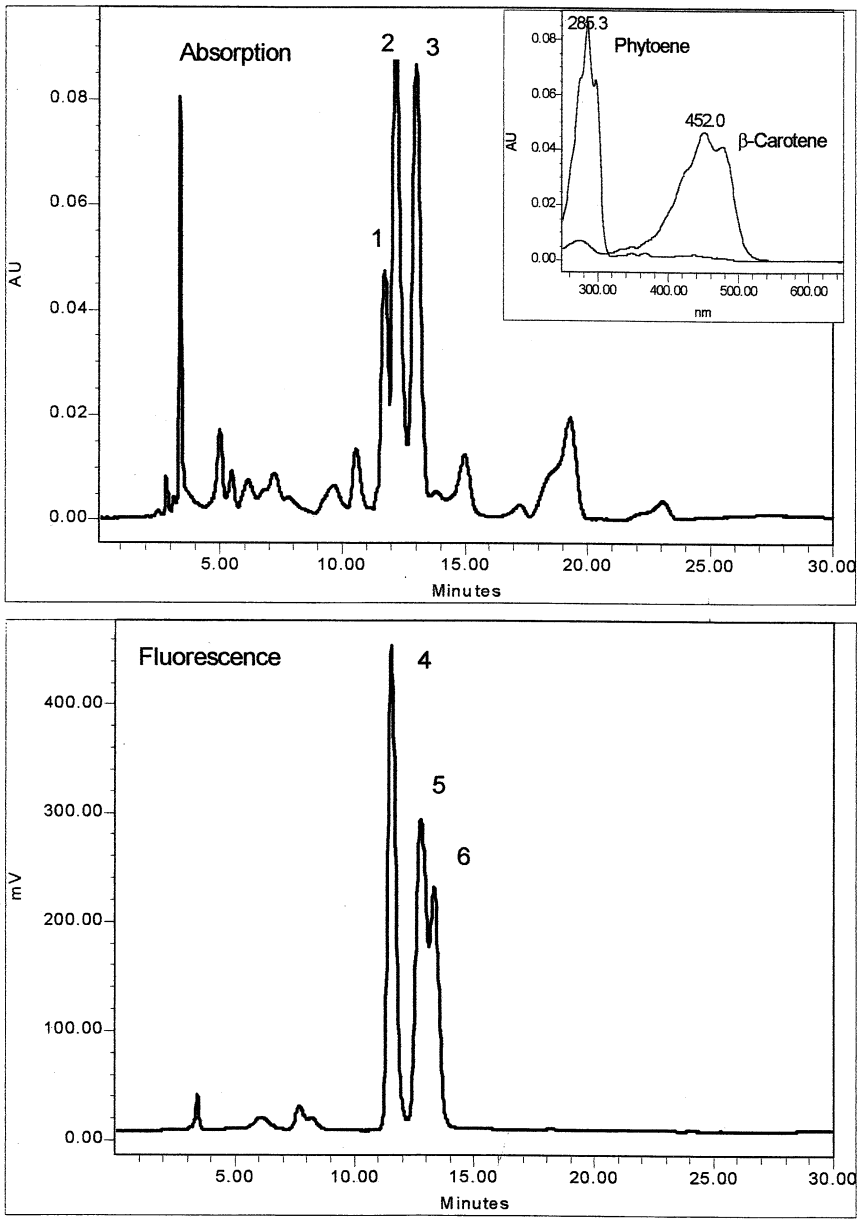

Fig. 1. High-pressure liquid chromatography (HPLC) profile of extract of the phytoene-rich *Dunaliella bardawil.* The following major components identified by their absorption maxima "Absorption" (upper figure) and by fluorescence emission "Fluorescence" (lower figure) are marked by numbers in parenthesis: *all-trans* phytoene at 285.3 nm (2), 9-*cis* phytoene at 285.3 nm (3), *all-trans* phytofluene at 348 nm and fluorescence emission at 520 nm (4). 9-*cis* phytofluene at 348 nm and fluorescence emission at 520 nm (5), *all-trans* β-carotene at 452.0 nm (1), not identified (6).

Table 1
Weight gain (g) and tissue weight (g/100 g BW) of rats fed on control diet, and on diet supplemented with either phytoene-rich *Dunaliella* or placebo*

	Control	Phytoene	Placebo
Body weight	71.15 ± 7.34	78.23 ± 4.51	72.33 ± 8.32
Liver	4.22 ± 0.45	4.18 ± 0.53	4.51 ± 0.55
Spleen	0.52 ± 0.23	0.43 ± 0.05	0.43 ± 0.04
Kidney	1.23 ± 0.09	1.31 ± 0.06	1.28 ± 0.11
Heart	0.48 ± 0.04	0.47 ± 0.04	0.48 ± 0.04
Lung	0.78 ± 0.09	0.73 ± 0.08	0.80 ± 0.10
Brain	1.48 ± 0.19	1.41 ± 0.09	1.46 ± 0.07

* Value are means ± SD.

Table 2
Hepatic concentration (μmol/100 mg wt) of phytoene, phytofluene, vitamin A and total carotenoids in rats fed on diet supplemented either with phytoene-rich *Dunaliella* or placebo (ND, not detected)*

	Phytoene	Placebo
Phytoene	0.440 ± 0.023	ND
Phytofluene	0.022 ± 0.002	ND
Vitamin A	0.053 ± 0.003	0.011 ± 0.002[a]
Total carotenoids	0.010 ± 0.001	Traces

* Values are means ± SD. Values in a row with[(a)] differ significantly ($P < 0.05$).

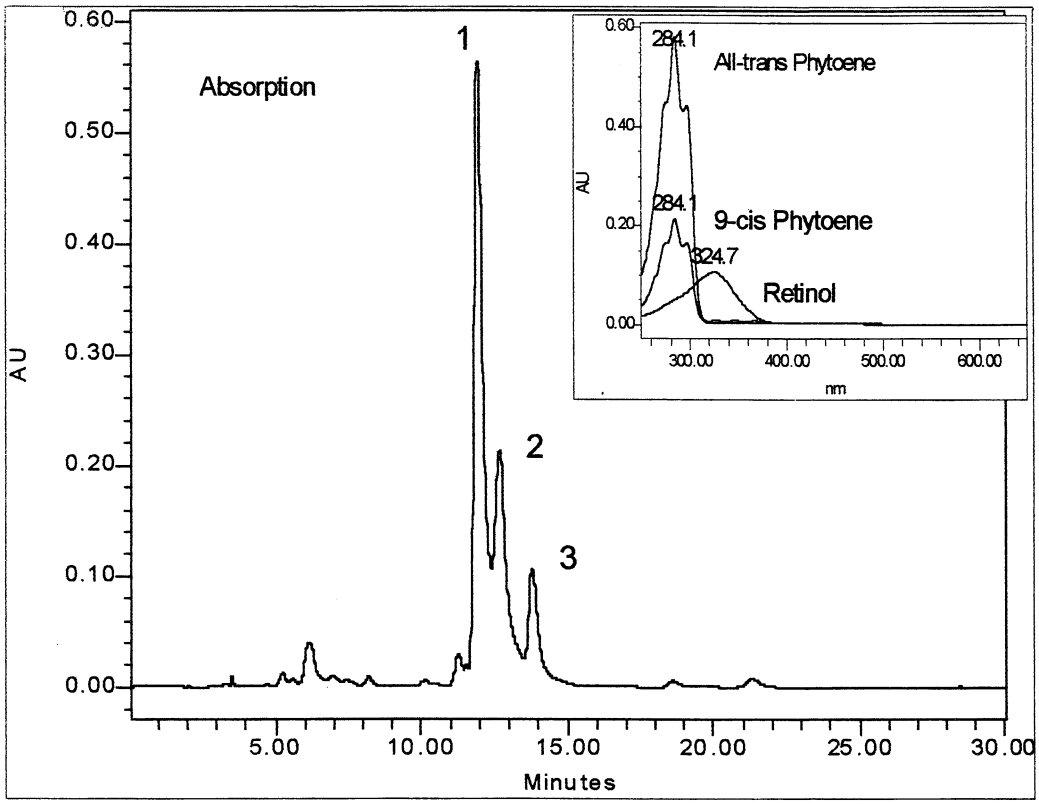

Fig. 2. High-pressure liquid chromatography (HPLC) max profile of liver extracts of animals fed on phytoene-rich *Dunaliella bardawil.* The following major components identified by their absorption maxima are marked by numbers in parenthesis: all-*trans* phytoene at 285.3 nm (1), 9-*cis* phytoene at 285.3 nm (2), retinol at 326 nm (3).

using fluorescence excitation at 350 nm and emission at 520 nm (Figure 1).

Growth performance and tissues weight of rats fed on basic diet and on basic diet supplemented with either the phytoene-rich *Dunaliella,* or its oxidized powder (placebo) showed no significant differences among the three studied groups (Table 1). The animals developed normally and the tissue weights were closely similar on the three different diets.

Hepatic pigment analysis of the experimental group revealed very high liver stores of phytoene, 0.44 μmol/100 mg wt, and normal levels of vitamin A, 0.053 μmol/100 mg, andtotal β-carotene, 0.01 μmol/100 mg (Table 2) [21–23]. The hepatic content of the placebo group showed low amount of vitamin A, and minimal contents of phytoene and β-carotene (Table 2). The liver pigments and vitamin A level of the placebo group fed on oxidized *Dunaliella* powder was basically similar to the control (not shown). Figures 2 and 3 present the HPLC stereoisomeric pigment absorption and fluorescence analysis of the liver extracts of animals fed on the phytoene-rich *Dunaliella bardawil.* The results demonstrate hepatic accumulation of phytoene and phytofluene with a ratio of 9-*cis*-to-*all-trans* phytoene in the liver close to 1:3 compared to 1:1 ratio in the algal supplemented diet.

Phytoene content was raised in a few other tissues of rats fed phytoene supplemented diet, significantly lower than the liver stores but higher than in the placebo (Table 3). It is of interest to note the high accumulation of phytoene in the adrenal, and less in spleen and in kidney. We have not detected measured amounts of phytoene in the other tissues, brain, heart and lung. The ratio between 9-*cis*-to-*all-trans* phytoene in the adrena, was 1:1, and in spleen and kidney was same to that observed in the liver, namely 1:3. The total phytoene content in the plasma was 0.012 μmol/ml (Table 3) with a 9-*cis*-to-*all-trans* ratio of 1:1, the same as the algal supplemented diet.

4. Discussion

Epidemiological investigations showed that cancer risk is inversely related to the consumption of green and yellow vegetables and fruits. Since β-carotene is present in abundance in these vegetables and fruits it has been investigated extensively as a possible cancer preventive agent. The properties of β-carotene as a potent free radical quencher, singlet oxygen scavenger and antioxidant, rather than its activity as pro-vitamin A, have been implicated as having major roles in this protective response. Numerous earlier studies dealt with the question of the protective role of dietary β-carotene against cancer development and the question remained on

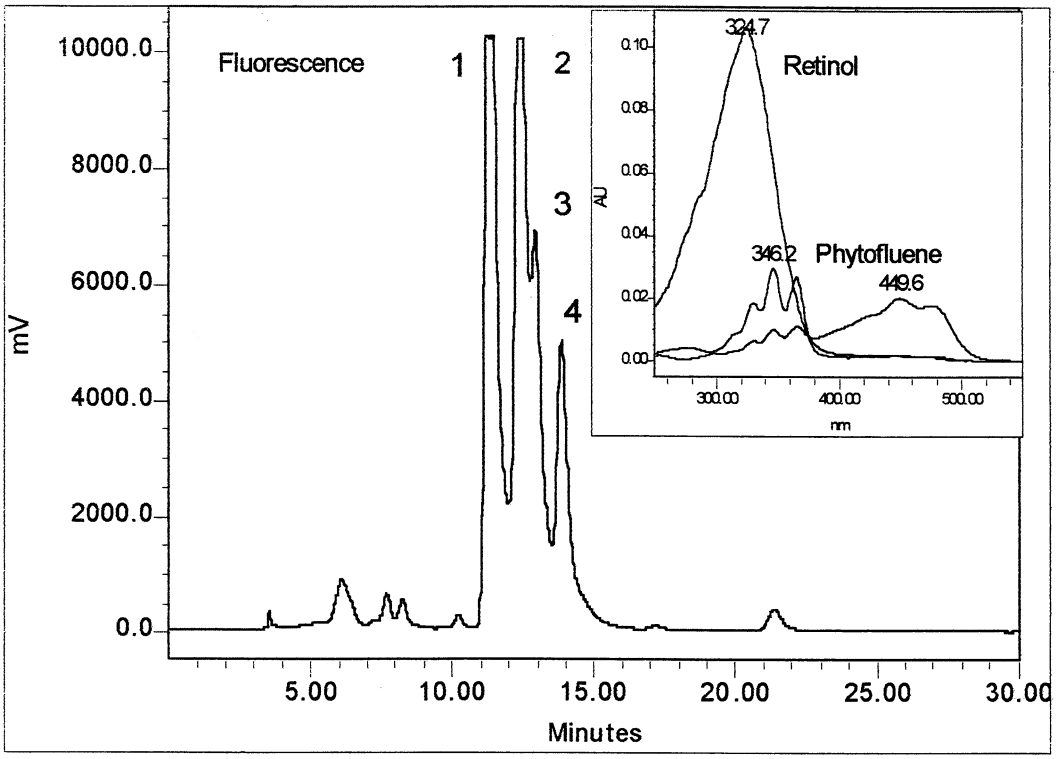

Fig. 3. High-pressure liquid chromatography (HPLC) max profile of liver extracts of animals fed on phytoene-rich *Dunaliella bardawil.* The following major components identified by fluorescence excitation at 350 nm and emission at 520 nm with photodiode array peak absorption are marked by numbers in parenthesis: 9-*cis* phytofluene (1), *all-trans* phytofluene (2&3), retinol (4).

debate until the publication of three large-scale intervention studies that clearly showed that not only did synthetic *all-trans* β-carotene fail to reduce the incidence of cancer, but in fact it significantly increased it and the related mortality in male smokers [4,6,7]. The reason for the carcinogenicity of *all-trans* β-carotene in human is not clear as yet, but it provoked a shift in the scientific attention to carotenoids others than β-carotene and their role in cancer prevention. A special attention was given to carotenoids of low or no pro-vitamin A activity such as γ-carotene, lycopene, lutein, zeaxanthin and canthaxantin. These compounds were shown to posses antioxidative properties which were related to their anticarcinogenic function [26]. Although the biological role of these carotenoids is not clearly defined, the literature includes studies on the metabolic fate of these compounds. Phytoene and phytofluene, colorless poly-isoprenoid hydrocarbons containing three and five double bonds are the first and second lipophilic precursors in the biosynthetic pathway of β-carotene in plants. Most fruits and vegetables contain phytoene and phytofluene but in small amounts relatively to the available colored carotenoids. Nishino [27] showed cancer preventing activity of phytoene and its antioxidative potential in mammalian cells. However, the literature lacks information on the metabolic fate of phytoene and phytofluene most probably due to the difficulty to supply edible product in sufficient amount to run statistical animal or human study.The alga *Dunaliella bardawil* was grown for the present study to contain large content of phytoene and phytofluene with equal amounts of the 9-*cis* and *all-trans* stereoisomers. The availability of large amount of digestible algal dry powder rich in stereoisomeric mixture of phytoene and phytofluene gave impetus to the present study on the absorption, storage and bioconversion of colorless carotenoids in animals.

In earlier studies we showed that low doses of the *Dunaliella* β-carotene were as potent as synthetic *all-trans* β-carotene in providing retinol in rats and chicks [21–23]. Preferential and selective uptake of *all-trans* more than 9-*cis* β-carotene was noted in serum in different animals and humans. 9-*cis* β-carotene was not detected in serum of chicks, rats and humans while the *all-trans* isomer was present. Rat liver does not accumulate β-carotene when provided in nutritional amounts and converts most of the dietary β-carotene into liver stored vitamin A [28]. Having substantial amounts of phytoene and phytofluene-rich *Dunaliella bardawil* in edible dry powder allowed us to study the fate of this non-pro-vitamin A colorless carotenoids and its stereoisomers in rat tissues.

The observation that weight gain and tissues weight of the rats fed on the control diet, or on the diet supplemented with either phytoene-rich *Dunaliella,* oxidized *Dunaliella,* or placebo were similar indicated that the experimented diets have the same nutritional value (bioavailability) with no side effect.

Table 3
Phytoene concentration of tissue (μmol/100 mg wt) and plasma (μmol/ml) and the stereoisomeric ratio of 9-cis/all trans phytoene of rats fed on diet supplemented either with phytoene-rich *Dunaliella* (ratio of 9-cis/all-trans = 1/1) or placebo (ND, not detected)*

	Phytoene	Phytoene stereoisomers (ratio of 9-*cis* to *all-trans*)	Placebo
Liver	0.440 ± 0.024	1/3	ND
Adrenal	0.017 ± 0.002	1/1	ND
Spleen	0.007 ± 0.001	1/3	ND
Kidney	0.005 ± 0.0007	1/3	ND
Brain	Traces		ND
Heart	ND		ND
Lung	ND		ND
Plasma	0.012 6.50 ± 0.002	1/1	ND
RBC	Traces		ND

* Values are means ± SD.

The hepatic pigment analysis clearly showed that the animals fed on the phytoene-rich diet accumulate and store phytoene in the liver. Due to the acyclic configuration of phytoene and the lack of literature information on its pro-vitamin A activity, it is reasonable to assume that this three double bond hydrocarbon is not converted to vitamin A in mammals. Calculation of μmol component in the liver peramount component in the diet shows that the bioavailability of phytoene and phytofluene is about the same and that of β-carotene is about one tenth as much. These findings support the notion that the increase of vitamin A levels is due to the conversion of 3 β-carotene, the most effective vitamin A precursor.

The higher vitamin A hepatic content over the placebo should originate from the conversion of the available algal β-carotene to vitamin A and as such the liver pigments and vitamin A content of the control group fed on oxidized *Dunaliella* powder was basically similar to the placebo. The HPLC stereoisomeric pigment analysis of the liver and the serum extracts demonstrated that the ratio of 9-*cis*-to-*all-trans* phytoene in the liver has different stereoisomeric composition compared to the 1:1 ratio in the algal supplemented diet. The original equal stereoisomeric composition was maintained in the serum and in the adrenal while the *all-trans* phytoene was enriched to give a ratio of 3**:**1, in liver, spleen and kidney. The preferential accumulation of *all-trans* phytoene over 9-*cis* phytoene in the liver, spleen and kidney may be interpreted as indicating stronger anti-oxidative effect of 9-*cis* phytoene over the *all-trans* isomer or alternatively, *in vivo* streoisomerization of 9-*cis* phytoene to the *all-trans* structure. The reduction in 9-*cis* phytoene in the liver but not in the adrenal may indicate direct transfer from the serum to the adrenal. Such reactions and pathways are suggested for β-carotene isomers [29], and may be relevant also to phytoene metabolism.

The abundance of phytoene in most plants is small but significant amounts are present in ripening tomatoes [9] and recently in different transgenic food and feed plants [30, 31]. Taking into consideration that cyclic and acyclic carotenoids are converted to cyclic and acyclic retinoids, respectively, and both play important role in gene activation through their activity as ligands to nuclear retinoic acid receptors, the interest in these compounds is recently increasing regarding human nutrition and medicine [32]. Although, carotenoids metabolism in rat is different than that of human, it is the most accepted animal available. This study opens the way to further research on the absorption of phytoene and phytofluene in mammals and in humans.

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