Carotenoids from native Brazilian dark-green vegetables are bioavailable: a study in rats

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Abstract

One of the major dietary sources of vitamin A in the human diet is provitamin A carotenoids. However, the activity of these carotenoids as a vitamin A source is uncertain due to concerns about the bioavailability of the carotenoids from vegetables. This study evaluated the bioavailability of provitamin A carotenoids from native Brazilian dark-green vegetables. Vitamin A–depleted rats were fed a basal diet (AIN-93G) in which the synthetic vitamin A content was replaced by non conventional leaves. At the end of the 30 day-repletion period, 1 µg of retinol accumulated in the liver after the intake of 43.1, 95.3 or 178.9 µg β-carotene from Sonchus oleraceus (So), Amaranthus viridis (Av) and Xanthosoma sagittifolium (Xs) leaves, respectively. The relative bioavailability of β-carotene from the leaves was 36 %, 16%, and 9% for So, Av, and Xs leaf, respectively. The results showed that the carotenoids from the three dark-green leaves were absorbed, converted to retinol, and stored in the liver of rats. Because they are pest-resistant and widely distributed vegetables, they may be an inexpensive alternative source of vitamin A to reduce vitamin A deficiency. © 2004 Elsevier Inc. All rights reserved.

Keywords: Bioavailability; Vitamin A; β-Carotene; Dark green leafy vegetables; Carotenoids

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1. Introduction

Nutritional vitamin A deficiency still continues to take lives, especially those of young children, in countries all around the world. Vitamin A deficiency, along with iodine and iron deficiencies, accounts for what is known as the “hidden hunger,” that afflicts various low-income communities in Latin America and the Caribbean region [1].

Preformed vitamin A is provided by food of animal origin; however, there are many provitamin A carotenoids from plants, which may be converted to retinol in mammalian cells. Dark-green leafy vegetables are rich in provitamin A carotenoids. However, the effectiveness of vegetables as a source of vitamin A has been questioned. De Pee et al. [2], in an interventional study, did not observe any improvement in the vitamin A status of children who received cassava leaves, water spinach, spinach, or carrots in their diet. Whereas, in a recent study, investigators observed that green and yellow vegetables maintained body stores of vitamin A in children [3]. The concern is about the bioavailability of carotenoids from vegetables, which is lower than that for pure carotenoids [4]. In fact, carotenoid bioavailability and bioconversion are influenced by different factors such as the species of carotenoids, molecular linkage, amount of carotenoids consumed in a meal, matrix of the food, nutrient status of the host, and genetic factors, among others [5].

*Amaranthus viridis* (slender amaranth), *Sonchus oleraceus* (smooth sow-thistle), and *Xanthosoma sagittifolium* (malanga) are vegetables rich in β-carotene. They are also easily cultivated, showing fast growth without any special care, and they are very resistant to pests [6,7]. Decades ago, these vegetables were widely cultivated and used by Brazil’s large rural population. However, nowadays, the majority of the Brazilian population is concentrated in urban areas, where these vegetables are not usually cultivated, and nor are they found in supermarkets. Therefore, these plants are considered nonconventional vegetables and are sometimes confused with harmful weeds in vegetable gardens [8,9]. Ironically, vitamin A deficiency still constitutes an endemic health problem in large areas of Brazil, and it is always associated with the limited ingestion of foods containing vitamin A [10].

Thus, the aim of the present study was to determine whether the carotenoids from these three vegetables serve as a potential vitamin A source. However, our bioavailability results from rats should not be directly extrapolated to humans because absorption and bioconversion may differ radically.

2. Methods and materials

2.1. Animals

A total of 43 male Wistar rats weaned at 21 days (46.11 ± 6.3 g) were purchased from BioAgri (Planaltina, D.F., Brazil). Rats were housed in individual cages in a room maintained at 22 ± 2°C with 12-hour light/dark cycle, and fed between 4 PM and 8 AM with free access to water. The Animal Care and Use Committee of the Universidade de Brasília in Brazil had approved the experimental protocol.
2.2. Leaves

The native Brazilian dark-green vegetables *Sonchus oleraceus* (smooth sow-thistle), *Amaranthus viridis* (slender amaranth), and *Xanthosoma sagittifolium* (malanga) were acquired from Toshiba-Nakamura LTDA, Brazlândia (D.F., Brazil). The vegetables were harvested in the winter, between 30 June and 30 July 2001. The leaves were weighed, washed with tap water, fried with soy oil for 2–3 minutes (according to their preparation by individuals who use these leaves as food), blended, and mixed in the AIN-93G diet to replace the pure \( \beta \)-carotene. The carotenoids from leaves were extracted and saponified according to Mercadante et al. [11] under N\(_2\) atmosphere. Carotenoid content was determined by HPLC using a Vydac polymeric C18 column, mobile-phase of 100% methanol [6], and the combined use of the retention time and 450 nm-absorption obtained with a photodiode array detector.

2.3. Dietary carotenoid composition

The diets supplemented with leaves had the following carotenoid composition. The *Xanthosoma sagittifolium* leaf diet contained 17,874.8 \( \mu \)g of total carotenoids (1,184.5 neoxanthin; 279.3 violaxanthin; 7,964.5 lutein; 98.4 \( \beta \)-cryptoxanthin; 1,727 \( \alpha \)-carotene; 5,442 \( \beta \)-carotene; and 1,179.1 cis-\( \beta \)-carotene \( \mu \)g/kg of diet). The *Amaranthus viridis* leaf diet contained 15,998 \( \mu \)g of total carotenoids (2,244 neoxanthin; 268.1 violaxanthin; 6,065.1 lutein; 67.4 \( \beta \)-cryptoxanthin; 145.8 \( \alpha \)-carotene; 5,969.7 \( \beta \)-carotene; 1,238 cis-\( \beta \)-carotene \( \mu \)g/kg of diet). The diet supplemented with *Sonchus oleraceus* leaves contained 13,437.3 \( \mu \)g of total carotenoids (2,444.2 neoxanthin; 5,185.9 lutein; 70.7 \( \beta \)-cryptoxanthin; 39.9 \( \alpha \)-carotene; 4,702.5 \( \beta \)-carotene; 994.1 cis-\( \beta \)-carotene \( \mu \)g/kg of diet) [6].

2.4. Experimental design

All rats were allowed to adapt for 5 days and given the complete AIN-93G diet [12] containing vitamin A. They were then weighed and four rats were killed to determine liver retinol. The remaining animals were given a vitamin A–deficient diet (–A): AIN-93G without any source of vitamin A, for 37 days to induce vitamin A deficiency (depletion period). At the end this depletion period, four rats were sacrificed to determine liver retinol. The remaining animals were distributed randomly into four groups. The control group \((n = 3)\), was treated with complete AIN-93G diet, replacing the vitamin A content by \( \beta \)-carotene, for 30 days (repletion period), and the deficient group received a deficient diet (–A), AIN-93G diet without any source of vitamin A. The other three groups \((n = 8)\) were treated during the 30-day repletion period with deficient diet supplemented with leaves in attempt to obtain similar amounts of \( \beta \)-carotene: diet \((Av)\), the deficient diet supplemented with 65.4 g/kg of *Amaranthus viridis* leaves; diet \((So)\), the deficient diet supplemented with 114 g/kg of *Sonchus oleraceus* leaves; or diet \((Xs)\) the deficient diet supplemented with 107 g/kg of *Xanthosoma sagittifolium* leaves. After the repletion period, the rats were killed by cervical dislocation and the liver was excised. The liver was washed in ice-cold saline, blotted on paper towels to remove excess blood and saline, and weighed. The liver was immediately
frozen in liquid N2 and stored at −70°C until the day of analysis. Food consumption was recorded daily and body weight once a week.

2.5. Retinol determination

The hepatic tissue (0.5 g) was first processed in a glass homogenizer, and then saponified for 40 minutes under reflux with 10 mL glycerin, 50 mL absolute ethanol, containing 0.125% BHT, and 10 mL of 5.3 mol/L potassium hydroxide. After cooling, the homogenate was transferred to a separatory funnel and washed with four volumes (40, 30, 20, and 10 mL respectively) of petroleum ether. The ether extracts were washed with water to remove excess potassium hydroxide, and filtered over anhydrous sodium sulfate to remove residual water. Afterward, the extract was evaporated to dryness in a rotatory evaporator, and the residue was redissolved in 2 mL of petroleum ether [13]. Retinol analysis was performed according to Furusho et al. [14] using the following HPLC conditions: Shim-park C18 25 cm CLC-ODS column, mobile-phase mixture of methanol/water (95:5), and a flow rate of 1.5 mL/min. Retinol was detected at 325 nm.

2.6. Determination of bioavailability

The bioavailability was based on Retinol Accumulation Factor (RAF) according to Zakaria-Rungkat et al. [15]. The RAF was calculated by dividing the β-carotene (RAF-β) or total carotenoid (RAF-C) intake by the total retinol accumulation in the liver (LRA). The relative bioavailability was determined by dividing the RAF-β from the control group (pure β-carotene) by RAF-β from each test group, multiplied for 100.

2.7. Statistical analyses

The data obtained for weight gain, diet consumption, carotene and carotenoid intake, and relative bioavailability were expressed as means ± standard deviation. Statistical analyses among the data from different groups were performed using analysis of variance with Bonferroni correction [16], with the Stats 95 program. Differences associated with \( P < 0.05 \) were regarded as significant. (The vegetables were acquired from agricultural farm Toshiba-Nakamura, Brozlandia city, Distrito Federal, Brazil.)

3. Results

The efficiency of the depletion period was shown in the depleted-rats that had a significant decrease in hepatic vitamin A content from 45 to 9.9 µg/liver. During this period, the rats showed an average weight gain of 194.24 ± 23.2 g. Vitamin A–depleted rats, when repleted with pure β-carotene (control diet) or with leaves of *Sonchus oleraceus* (So), *Amaranthus viridis* (Av), or *Xanthosoma sagittifolium* (Xs) gained weight equally. In contrast, the growth of vitamin A–depleted rats was significantly lower (\( P = 0.028 \)) than that of the control group (Table 1).
Table 1 shows the accumulated amount of food consumed during the repletion period (30 days). The rats showed equal food consumption except for the group repleted with (So) leaves, which consumed a little less diet; this difference was significant (P < 0.032). The (Av) leaves intake of the rats varied from 2,445.1 for (So) leaves to 3,304.7 g for (Av) leaves, respectively. Likewise, the amount of total carotenoids consumed varied from 2,976.9 g for (So) leaves to 4,591.5 g for (Xs) leaves (P < 0.05).

Among the rats on test diets, including the rats not subjected to vitamin repletion, no difference was observed in liver weight (12.1–13.9 g) at the end of the study (Table 1). However, the control rats showed a higher value than the other rats (18.4 ± 2.4 g). At the end of the repletion period, about 99 % of the liver retinol reserve was depleted in the animals fed a vitamin A–deficient diet, whereas all the other groups showed an increase in liver retinol content (Table 2), independent of the type of vegetable carotenoid source. On the other hand, the liver retinol accumulation differed greatly from that of the control and the other repleted groups (Table 2). In fact, the LRA value of 310.8 μg/liver from rats fed (Av)-carotene was about 5–18 times higher than that of the test groups (P < 0.03).

Table 2

<table>
<thead>
<tr>
<th>Diet</th>
<th>β-Carotene Intake (μg)</th>
<th>Carotenoid Intake (CI)* (μg)</th>
<th>Liver Retinol Accumulation LRA (μg)</th>
<th>RAF-β† (βI/LRA)</th>
<th>RAF-C† (CI/LRA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(−A)</td>
<td>0</td>
<td>0</td>
<td>0.4 ± 0.1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>(Av)</td>
<td>3304.7 ± 269.2b</td>
<td>4142.1 ± 342.5b</td>
<td>34.7 ± 7.4b</td>
<td>95.3b</td>
<td>119.5c</td>
</tr>
<tr>
<td>(So)</td>
<td>2445.1 ± 322.6c</td>
<td>2976.9 ± 415.6c</td>
<td>56.8 ± 13.2b</td>
<td>43.1c</td>
<td>52.4d</td>
</tr>
<tr>
<td>(Xs)</td>
<td>2963.7 ± 290.5b</td>
<td>4591.5 ± 446.1a</td>
<td>16.7 ± 2.7d</td>
<td>178.9a</td>
<td>277.1a</td>
</tr>
<tr>
<td>Control</td>
<td>4634.6 ± 785.9a</td>
<td>0</td>
<td>310.8 ± 94.3a</td>
<td>15.4d</td>
<td>0</td>
</tr>
</tbody>
</table>

* Considered all provitamin A carotenoids present in each vegetable leaves.
† RAF-β and RAF-C = retinol accumulation factor for β-carotene and provitamin, respectively.

Values in columns not sharing a common superscript letter are significantly different (P < 0.03).

All groups, n = 8, except Control group, for which n = 3.

See Table 1 for diet abbreviations.
In the present study, RAF expressed the bioavailability of carotenoids. The RAF-β-carotene (RAF-β) and RAF-provitamin carotenoid (RAF-C) were obtained according to Zakaria-Rungkat et al. [15] by dividing the β-carotene or provitamin A carotenoid intake (μg) by the liver retinol accumulation–LRA (μg) for each test diet consumed during the repletion period. The RAF-β average calculated for pure β-carotene from the control group was of 15.4, which means that 15.4 μg of the β-carotene intake produced 1 μg liver retinol. The lowest RAF value reflects the highest bioavailability. RAF values of β-carotene from the vegetable leaves were lower than the RAF-β for pure β-carotene (P < 0.0001). Among the vegetable test diets, the lowest RAF–β found was 43.1 for (So) leaves followed by that for (Av) and (Xs) leaves, which was 95.31 and 178.9 respectively. Based on the total content of provitamin A carotenoids in the leaves, determined by HPLC, namely β-carotene, α-carotene, cis-β-carotene, and β-cryptoxanthin, the RAF-C values were close to the RAF-β results. Hepatic retinol (1 μg) could be obtained from the ingestion of 52.4, 119.5, and 277.1 μg provitamin A carotenoids from (So), (Av), and (Xs) leaves, respectively.

4. Discussion

The purpose of the present study was to measure the bioavailability of provitamin A carotenoids from native Brazilian dark-green vegetables. To attain this goal, vitamin A–depleted rats were fed a basal diet (AIN-93G) replacing the synthetic vitamin A with leaves from Sonchus oleraceus (So), Amaranthus viridis (Av) and Xanthosoma sagittifolium (Xs). The leaves had been prepared according to local recipes, following instructions provided by individuals who use these leaves as food; however; it is not our intention to extrapolate the present results directly to humans.

Vitamin A deficiency in young animals may result in growth impairment, abnormal bone formation, degeneration of the reproductive organs, and epithelial keratinization [17,18]. During the depletion period, the mean growth rate was found to be 5.3 g/day, and thus very close to the 5.6 g/day value described by Kohn et al. [19] as the normal rate. This result suggests that vitamin A deficiency did not impair growth rate during the depletion period, probably because the retinol stored in the liver during breast-feeding and the acclimation period provided the vitamin A necessary to maintain normal rat growth. However, during the vitamin repletion phase, the growth rate observed in the deficient rats (2.6 g/day) was significantly lower than in the control group (3.7 g/day). Earlier studies have suggested that marginal vitamin A status is not severe enough to impair growth [20]. In fact, Lewis et al. [21] provided quantitative and descriptive evidence of an efficient metabolism of vitamin A from absorption, through turnover and utilization, in rats with a very low vitamin A status. Nevertheless, in the present study, vitamin A deficiency did affect the growth of rats.

The animals repleted with the dark-green vegetables showed a retinol accumulation higher than the deficient group (P < 0.004), providing evidence for the bioavailability of carotenoids from the native dark-green vegetables.

There was no direct correlation between β-carotene intake and liver retinol content, since the animals repleted with (So) leaves, which provided the lowest β-carotene intake, showed the highest liver content among the vegetable groups. In fact, the effectiveness of the three
native vegetables on the increase in hepatic retinol differed substantially. In addition, the carotenoid composition of the leaves was not similar; the (Av) leaves had the highest β-carotene concentration (9,128 μg/100 g), whereas the (Xs) leaves had the largest amount of α-carotene (1,614 μg/100 g).

Furosho et al. [14] have shown that the retinol equivalence of carotenoids can be determined by hepatic vitamin A content. Recently, Zakaria-Rungkat et al. [15] proposed the use of a retinol accumulation factor (RAF) to measure bioavailability, considering that the retinol accumulated in liver is derived from β-carotene (RAF-β) or total carotenoids (RAF-C) from the diet. As evident from other studies, our results showed that the bioavailability of pure β-carotene was higher than that of β-carotene from vegetables, since the RAF-β values found for the leaves were lower than the RAF-β for pure β-carotene. Among the vegetables, the highest bioavailability was found for (So) leaves, with values of 43.1 and 52.4 for RAF-C and RAF-β, respectively. In other words, the ingestion of 43.1 μg of β-carotene or 52.4 μg of provitamin A carotenoids from (So) leaves resulted in the presence of 1 μg of retinol in the liver.

Carotenoid bioavailability is influenced by multiple factors such as carotenoid species, matrix properties, and carotenoid interactions during the absorption, metabolism, and transport process [22]. In the present study, the β-carotene bioavailability of leaves was significantly improved by frying in soy oil and blending them before mixing with diet, as described in the literature [22]. Although the literature has indicated that the lutein may reduce β-carotene bioavailability [23], our results indicated that differences in bioavailability might not be attributed to lutein, since all the test diets had a similar proportion of β-carotene:lutein (1:1). Thus, further studies are warranted to elucidate the mechanisms involved in carotenoid bioavailability observed with native Brazilian vegetables.

As expected, the bioavailability based on the RAF-β was higher than the bioavailability found for provitamin A carotenoids (RAF-C), since the other vitamin A precursors are less biologically active than β-carotene. All the RAF-β values calculated for the leaves in the present study were close to the RAF-C values, varying from 0.21 to 0.54 times. In contrast, the RAF-C and RAF-β values from fruits and vegetables reported originally by Zakaria-Rungkat et al. [15] showed a larger variation from 0.8 to 10.5 times. The proximity of our the RAF-C and RAF-β values can be attributed to the fact that in the vegetables analyzed in the present study, β-carotene was the most abundant precursor (65–81 %). Moreover, we considered for the calculation of RAF-C just the provitamin A carotenoids. Therefore, the knowledge of the specific carotenoid in the food is an indispensable condition for the determination of bioavailability using the factor RAF-C.

Cooking, in general, increases the availability of carotenoids, probably by the disruption of plant cell walls and breaking the protein-carotenoids complexes [5]. Even with frying and blending of the leaves, we found bioavailability values significantly lower than for pure β-carotene incorporated in the same basal diet (control group).

The relative bioavailability of β-carotene from plants is as low as 5–8% compared to pure β-carotene; this is attributed to several diet factors such as the carotenoid species in the food, the matrix in which carotenoid occurs, and the composition of the consumed diet [2,4]. However, Huang et al. [24] recently found a higher value of 25% for the relative bioavailability of β-carotene from stir-fried water convolvulus leaves. In the present study, the
relative bioavailability of β-carotene from (So), (Av), and (Xs) was about 36%, 16%, and 9% respectively. These values were close to that for the relative bioavailability of provitamin A carotenoid (~30%, 13%, and 6%, respectively).

The bioavailability of vitamin A from carotenoid precursors in fruits and vegetables has been studied largely in commercially grown produce, since it constitutes the main source for most of the human population, not only in developed countries. Various dark-green leafy vegetables that are home grown are used as part of the daily food of many individuals, however, especially those of low income. For these individuals, the provitamin A carotenoids present in these vegetables contribute 70–90% of the total vitamin A consumed [5]. Therefore, further investigations into the potential of native Brazilian dark-green vegetables as vitamin A sources should be encouraged, before the disappearance of these carotenoid-rich vegetables or their complete substitution by crops that may be more expensive and poorer in vitamin precursors.

In conclusion, this study shows that the provitamin A carotenoids from the Brazilian dark-green vegetables Sonchus oleraceus, Amaranthus viridis, and Xanthosoma sagittifolium were effective in improving vitamin A status. The bioavailability calculated for β-carotene was very close to that for the provitamin A carotenoids, since β-carotene was the precursor most abundant in these vegetable leaves. Bioavailability varied substantially among the vegetables, with the highest relative bioavailability of β-carotene being from Sonchus oleraceus (36%), followed by Amaranthus viridis (16%), and Xanthosoma sagittifolium (9%). Provitamin A carotenoid bioavailability should be further evaluated to prompt public policy aimed at the use of these native vegetables to improve nutritional status in needy populations.

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