Determination of bioaccessibility of β-carotene in vegetables by *in vitro* methods

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The *in vitro* method in use for the determination of β-carotene bioaccessibility involves simulated gastrointestinal digestion followed by ultracentrifugation to separate the micellar fraction containing bioaccessible β-carotene and its quantitation. In this study, the suitability of two alternatives *viz.*, membrane filtration and equilibrium dialysis were examined to separate the micellar fraction. Values of β-carotene bioaccessibility obtained with the membrane filtration method were similar to those obtained by the ultracentrifugation method. Equilibrium dialysis was found not suitable for this purpose. Among the vegetables analyzed, fenugreek leaves had the highest content of β-carotene (9.15 mg/100 g), followed by amaranth (8.17 mg/100 g), carrot (8.14 mg/100 g) and pumpkin (1.90 mg/100 g). Percent bioaccessibility of β-carotene ranged from 6.7 in fenugreek leaves to 20.3 in carrot. Heat treatment of these vegetables by pressure cooking and stir-frying had a beneficial influence on the bioaccessibility of β-carotene from these vegetables. The increase in the percent bioaccessibility of β-carotene as a result of pressure-cooking was 100, 48 and 19% for fenugreek leaves, amaranth and carrot, respectively. Stir-frying in presence of a small quantity of oil led to an enormous increase in the bioaccessibility of β-carotene from these vegetables, the increase being 263% (fenugreek leaves), 192% (amaranth leaves), 63% (carrot) and 53% (pumpkin).

Keywords: Bioaccessibility / β-Carotene / Green leafy vegetables / Heat processing / Yellow-orange vegetables

Received: May 25, 2006; revised: July 8, 2006; accepted: July 10, 2006

1 Introduction

Micronutrient deficiency is a major public health problem in the developing countries, India accounting for nearly half of the world's prevalence (Micronutrient Deficiency Information System. Global Prevalence of Vitamin A deficiency. MDIS Working Paper # 22 WHO Geneva, 1995). Among the micronutrient deficiencies, vitamin A deficiency is recognized as a serious public health problem leading to blindness. It has been estimated that globally, 2.8 million preschool children are at risk of blindness [World Health Organization (1998) Studies Rebut Concept that body stores vitamin A making substance, pp 1–2]. Deficiency of vitamin A is wide spread in India leading to the blindness of about 60 000 children below the age of 5 years, annually. Animal foods such as eggs, milk and liver are good sources of preformed vitamin A. A majority of the population in India is dependent on plant foods, which provide carotenes, especially β-carotene, to meet their requirement of vitamin A. β-carotene is abundantly found in green leafy and yellow-orange vegetable [1]. Several factors such as diet composition and methods employed for food processing affect the bioaccessibility of β-carotene from foods. Dietary factors such as fat, fiber and protein are documented to influence β-carotene bioaccessibility [2]. Earlier investigations have evidenced that inclusion of phospholipids and certain specific fatty acids in the diet significantly improve the vitamin A status of experimental animals [3, 4]. Studies have shown that the absorption of carotenoids from uncooked food is low and mild cooking enhances the absorbability of β-carotene [5]. However, heat treatment especially in presence of light and oxygen causes isomerization of carotene as well as its oxidative destruction thus decreasing its biological activity [6].

Vitamin A malnutrition being widely prevalent, understanding the bioaccessibility of dietary β-carotene from plant foods is of utmost importance. Such information may

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lead to optimization of dietary approaches to increase the bioaccessibility of dietary β-carotene. Knowledge of the bioaccessibility of micronutrients including β-carotene from dietary sources is also important in order to rationalize the recommended daily allowance (RDA) for vitamin A. *In vitro* methods to determine the bioaccessibility of β-carotene from foods appear to provide a cost effective alternative to the more expensive and cumbersome *in vivo* procedures.

Recently, an *in vitro* digestion method to estimate carotenoid bioaccessibility from meals similar to the one employed for determination of iron bioaccessibility was developed by Garrett *et al*. [7]. This method essentially involves simulated gastrointestinal digestion followed by ultracentrifugation to separate the micellar aqueous fraction containing the bioaccessible fraction of β-carotene and determination of the same. In the present study, alternative methods were explored to separate the micellar fraction containing the bioaccessible β-carotene after simulated gastrointestinal digestion. These alternative methods could be simpler, faster, more economical and suitable for routine screening of plant foods for β-carotene bioaccessibility. These methods were employed to determine the bioaccessibility of β-carotene from two representative green leafy and yellow-orange vegetables. The influence of two common domestic heat treatment processes, namely, pressure-cooking and stir-frying on the bioaccessibility of β-carotene from these vegetables was also studied.

2 Materials and methods

2.1 Materials

Fresh carrot (*Daucus carota*), pumpkin (*Cucurbita maxima*), amaranth (*Amaranthus gangeticus*) leaves and fenugreek (*Trigonella foenum-graecum*) leaves were locally procured. All chemicals used were of analytical grade. Solvents were distilled before use. Porcine pancreatic pepsin, and pancreatin and bile extract (porcine) were procured from Sigma Chemicals (St. Louis, MO). Double-distilled water was employed through out the entire study. All glassware used was acid washed.

2.2 Determination of bioaccessibility of β-carotene *in vitro*

The bioaccessibility of β-carotene *in vitro* was determined by the method of Garrett *et al.* [7]. Briefly, the method involved subjecting the sample to simulated gastric digestion at pH 2.0 in the presence of pepsin at 37°C (16 g in 100 mL 0.1M HCl), followed by simulated intestinal digestion in the presence of pancreatin-bile extract mixture (4 g porcine pancreatin) and 25 g by bile extract (porcine) in 1000 mL of 0.1M $NaHCO_3$ pH 7.5 at 37°C for 2 h. At the end of simulated intestinal digestion, the micellar fraction, containing the bioaccessible β-carotene, was separated by ultracentrifugation at 70 000 × *g* for 120 min using a Beckman L7-65 ultracentrifuge (method 1). An alternative method to separate the micellar fraction examined for its suitability in this investigation was filtration of the digesta through a Millipore membrane (0.65 μm pore size; 25 mm diameter) after a preliminary centrifugation at 5000 × *g* for 20 min using polyallomer tubes (40 mL capacity) in a SS-34 rotor and Sorvall RC-5B super speed refrigerated centrifuge, instead of ultracentrifugation (method 2).

Another alternative method to separate micellar fraction examined for its suitability in this investigation was equilibrium dialysis by insertion of a dialysis bag containing sodium bicarbonate equimolar to the titratable acidity, and mixed micelles to facilitate the movement of the lipid-soluble β-carotene into the dialysis bag, during simulated intestinal digestion (method 3). During simulated intestinal digestion, segments of dialysis tubing (molecular mass cut-off 10 kDa) containing 25 mL sodium bicarbonate solution, being equivalent in moles to the NaOH needed to neutralize the gastric digest (titratable acidity) were placed in Erlenmeyer flasks containing the gastric digest and incubated at 37°C with shaking for 30 min or longer until the pH of the digest reached 5.0. The pancreatin-bile extract mixture (5 mL) was added and incubation was continued for 2 h or longer until the pH of the digest reached 7.0. Mixed micelles were prepared using a mixture of phosphatidyl choline and deoxycholic acid in the molar ratio 1 : 2 as described by Began *et al*. [8]. Solutions of phosphatidyl choline and deoxycholic acid were made in chloroform-methanol (2 : 1); after mixing the two solutions, the solvent was evaporated in a flash evaporator and dried under a stream of nitrogen. The resulting thin film was solubilized in 50 mM Tris-HCl buffer, pH 7.4 by sonication for 5 min in a bath type sonicator.

The concentration of mixed micelles (prepared as described above) taken in the dialysis bag along with sodium bicarbonate (equivalent of titratable acidity) was 100 μM in terms of phosphatidyl choline. This amount was optimized in a preliminary trial, by measuring the amount of β-carotene from raw amaranth dialyzed as a function of increasing concentrations of the mixed micelles in the dialysis bag (Fig. 1). The amount of β-carotene that was dialyzed from the digesta of amaranth, which was insignificant in the absence of any mixed micelles, increased with the inclusion of the same in the dialysis bag. Dialyzability of β-carotene increased linearly with increasing micellar concentration in the dialysis bag up to 100 μM (in terms of phosphatidyl choline). Further increase in micellar concentration did not proportionately improve β-carotene dialyzability. On the

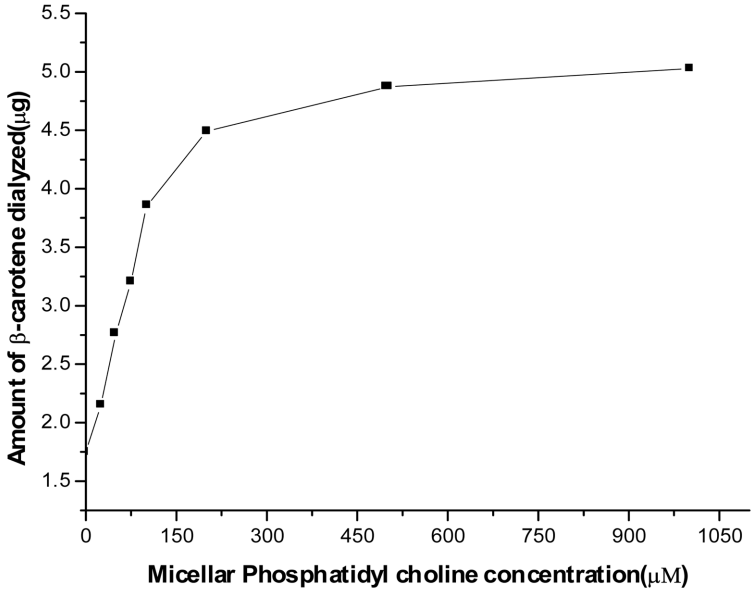

Figure 1. Dialyzability of β-carotene from the digesta as a function of micellar phosphatidyl choline concentration in the dialysis bag.

other hand, increase in micellar concentration beyond 100 μM also resulted in an undesirable turbidity of the dialysate. Thus, micellar concentration in the dialysis bag was limited to 100 μM in terms of phosphatidyl choline in all determinations involving method 3.

2.3 Analysis of β-carotene

Total as well as bioaccessible β-carotene were determined by extraction of the pro-vitamin from the samples with acetone followed by petroleum ether (60 – 80°C), and fractionated on neutral alumina using 3% acetone in petroleum ether. The color intensity of β-carotene eluent was measured at 450 nm in a Shimadzu UV/Visible spectrophotometer, and compared with that of β-carotene standard [9]. In methods 1, 2 and 3, β-carotene was quantitated from the micellar fraction as well as the residue, and the bioaccessibility of β-carotene expressed as a percentage of this recovered amount.

During the entire procedure, namely simulated gastrointestinal digestion, ultra-centrifugation/membrane filtration, extraction of β-carotene and column chromatography, adequate precautions were taken to minimize the exposure of samples to light and air and thus prevent oxidative destruction of β-carotene. Air was replaced by nitrogen before stoppering the flask at all stages of incubation and storage. The experiments were carried out under yellow lighting and all glassware was covered with black cloth to prevent penetration of light.

2.4 Heat processing of food samples

Two methods of heat processing, namely, pressure-cooking and stir-frying were employed in the study. For pressure-cooking, 2 g of the vegetable sample was cooked with 10 mL of distilled water at 15 psi for 10 min. In the case of stir-frying, 2 g of chopped vegetable samples were fried in a shallow pan in the presence of 185 mg of refined groundnut oil for 10 min at 100°C. The heat-processed samples were homogenized before being subjected to simulated gastrointestinal digestion.

2.5 Statistical analysis

All determinations were made in pentuplicates and the average values are reported. Data were analyzed statistically according to Snedecor and Cochran [10].

3 Results and discussion

3.1 Total β-carotene content of the test vegetables

Table 1 presents the total β-carotene content of the four test vegetables, carrot, pumpkin, amaranth and fenugreek leaves. Fenugreek had the highest concentration of β-carotene (9.15 mg/100 g), followed by amaranth (8.17 mg/100 g), carrot (8.14 mg/100 g) and pumpkin (1.90 mg/100 g). These values are comparable to those reported by the Indian Council of Medical Research [1], where the β-carotene content of fenugreek leaves, amaranth, carrot

and pumpkin are reported as 9.10, 8.34, 6.46 and 1.17 mg/100 g, respectively.

3.2 Comparison of three methods to determine bioaccessible β-carotene

Figure 2 presents a comparison of the bioaccessibility of β-carotene from the four test vegetables as determined by the three methods described above. Bioaccessible β-carotene was calculated as the percentage of the total amount recovered in the residue and micellar aqueous fractions, at the end of simulated gastrointestinal digestion. The amount of β-carotene recovered from these two fractions by methods 1 and 2 was 72 – 85% of that of the initial value in the unprocessed vegetables, while the same was between 61 and 86% by method 3. Such losses in the recovery of β-carotene during prolonged processing procedures are to be expected, as this provitamin is highly susceptible to destruction by exposure to light and oxygen. Among the alternatives tried for method 1, method 2, which involved membrane filtration to separate the micellar fraction, produced values for bioac-

Table 1. Influence of heat processing on the bioaccessibility of β-carotene from test vegetables

Vegetable		β-Carotene content (mg/100 g)	Percent bioaccessible β-carotene	
			Method 1[a)]	Method 2[a)]
Carrot	Raw	8.14 ± 0.14	20.3 ± 0.58	16.5 ± 0.03
	Pressure-cooked		24.2 ± 0.38[b)] (19%)	21.0 ± 0.35[b)] (27%)
	Stir-fried		32.9 ± 0.09[b)] (63%)	28.0 ± 0.40[b)] (70%)
Pumpkin	Raw	1.90 ± 0.27	16.3 ± 0.84	19.0 ± 0.17
	Pressure-cooked		15.3 ± 0.64 (0%)	19.0 ± 0.09 (0%)
	Stir-fried		24.9 ± 1.18[b)] (53%)	20.9 ± 0.64[b)] (10%)
Amaranth	Raw	8.17 ± 0.54	10.6 ± 0.58	11.0 ± 0.49
	Pressure-cooked		15.7 ± 0.32[b)] (48%)	16.5 ± 0.87[b)] (50%)
	Stir-fried		30.6 ± 1.20[b)] (192%)	23.5 ± 0.06[b)] (114%)
Fenugreek leaves	Raw	9.15 ± 0.05	6.70 ± 0.23	7.10 ± 0.29
	Pressure-cooked		13.4 ± 0.06[b)] (100%)	15.2 ± 0.26[b)] (114%)
	Stir-fried		24.3 ± 1.04[b)] (263%)	21.5 ± 0.06[b)] (203%)

a) Method 1: Ultracentrifugation to separate micellar fraction; Method 2: Membrane filtration to separate micellar fraction. Values are mean ± SEM of pentuplicate determinations; figures in parentheses indicate the percent increase in bioaccessible β-carotene as compared to the raw value.

b) Statistically significant increase ($p < 0.05$) compared to the corresponding value of the raw sample.

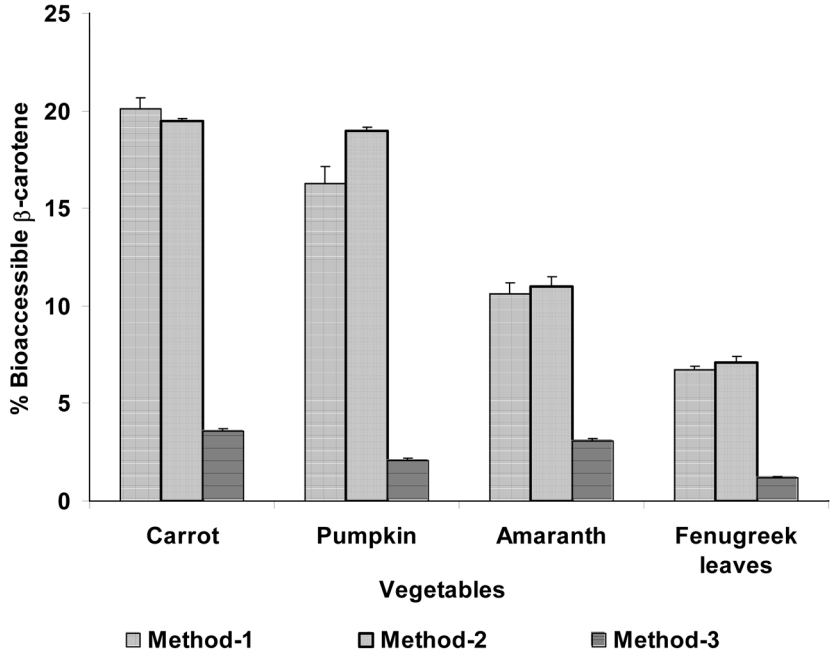

Figure 2. Bioaccessibility of β-carotene as determined by three variations of the *in vitro* procedure: Method 1: ultracentrifugation to separate micellar fraction; method 2: membrane filtration to separate micellar fraction; and method 3: equilibrium dialysis.

cessible β-carotene that were comparable with those of method 1 for all test vegetables examined. On the other hand, method 3, where the bioaccessible fraction of β-carotene was separated by equilibrium dialysis, gave values that were several-fold lower than those obtained by methods 1 and 2.

The percent bioaccessible β-carotene from raw carrot was 20.3 as determined by method 1, while it was 19.5 by method 2 and 3.58 by method 3. The bioaccessiblity of β-carotene from raw pumpkin was 16.3, 19.0 and 2.1% as determined by methods 1, 2 and 3, respectively. Among the green leafy vegetables tested, the bioaccessibility of β-carotene from raw amaranth as determined by method 1 was 10.6%, while it was 11.0 and 3.10% by methods 2 and 3, respectively. The percent bioaccessible β-carotene from raw fenugreek leaves was 6.7, 7.1 and 1.2 as obtained by methods 1, 2, and 3, respectively. Thus, membrane filtration to separate the bioaccessible fraction of β-carotene after simulated gastrointestinal digestion, has provided values for bioaccessible β-carotene similar to those obtained by ultracentrifugation.

On the other hand, the values obtained by method 3, which involved equilibrium dialysis, were lower than those obtained by the other two methods. In this method, the migration of the bioaccessible fraction of β-carotene, a lipid soluble provitamin, from the gastric digesta into the dialysis tube may have been restricted by the fact that the tubing contains an aqueous solution of sodium bicarbonate. Although the amount of β-carotene that was dialyzed from the digesta linearly increased with the inclusion of mixed micelles up to 100 μM (in terms of phosphatidyl choline) in the dialysis bag, addition of mixed micelles at the level used here to the tubing did not result in β-carotene dializability comparable to the micellar β-carotene found with the other two methods. Membrane filtration after a preliminary low speed centrifugation to separate the bioaccessible fraction of β-carotene may be considered as a suitable alternative to ultracentrifugation. This alternative method may be less expensive, and can probably be used when the availability of an ultracentrifuge is a limitation. On the other hand, simulated gastrointestinal digestion involving equilibrium dialysis, an *in vitro* method employed in the determination of mineral bioaccessibility, is not suitable for the determination of the bioaccessibility of the lipid-soluble β-carotene. The use of mixed micelles in this method at an appropriate higher concentration would also prove expensive.

3.3 Bioaccessibility of β-carotene from raw and heat processed vegetables

Table 1 presents data on the bioaccessibility of β-carotene from the raw and heat-processed vegetables, as determined by methods 1 and 2. Percent bioaccessibility of β-carotene in the raw vegetables ranged from 6.7 in fenugreek leaves to 20.3 in carrot. In general, the fleshy vegetables had a higher content of bioaccessible β-carotene, as compared to the leafy vegetables. Hedren *et al*. [11, 12] reported values of 21 and 18%, respectively, for the bioaccessiblity of β-carotene from carrot and amaranth leaves. The values obtained in the present study are comparable to these reported values. The values reported for other leafy vegetables, that is, cooked sweet potato leaves and cooked pumpkin leaves are 9 and 10%, respectively [12]. The value obtained in our study for the flesh portion of the pumpkin (16.3%) is thus higher than that reported for pumpkin leaves. Thus, carrot provides more bioaccessible β-carotene than any other fleshy vegetable.

Heat processing is believed to improve the bioaccessibility of β-carotene by loosening the matrix and thus facilitating its absorption. In this study, we have examined the influence of two types of heat processing, viz. pressure-cooking and stir-frying, generally employed in Indian culinary practices. Table 1 presents β-carotene bioaccessibility values from the four vegetables subjected to these two heat-processing methods. Both heat-processing treatments had a significant enhancing influence on the bioaccessibility of β-carotene from all vegetables examined. The extent of increase in the percent bioaccessibility of this provitamin as a result of pressure-cooking was 100, 48 and 19% from fenugreek leaves, amaranth and carrot, respectively. Pressure-cooking did not have any influence on the bioaccessibility of β-carotene from pumpkin.

Stir-frying the vegetable in the presence of a small quantity of oil (9% w/w) brought about an enormous increase in the bioaccessibility of β-carotene from all the four vegetables examined, the extent of increase being 263% (fenugreek leaves), 192% (amaranth leaves), 63% (carrot) and 53% (pumpkin). The oil *per se* did not contribute to the β-carotene content of the sample. It is well known that presence of fat improves the bioaccessibility of β-carotene, which is lipophilic [2]. Similar increases in the bioaccessibility of β-carotene from carrot and amaranth as a result of heat processing in the presence of oil have been reported in other studies. An *in vivo* study conducted by Huang *et al.* [13] reported bioaccessibility of 33% from carrot stir-fried in presence of 15% oil. The *in vitro* accessibility of β-carotene from carrot, cooked in the presence of oil (2%) was found to be 39% [11]. Thus, stir-frying could offer a good strategy to derive maximum amounts of bioavailable β-carotene from dietary sources. While the bioaccessibility of β-carotene was higher from raw fleshy vegetables, heat processing brought about a higher increase in the same from the leafy vegetables. This difference could be due to the differences in the alteration of the matrices of these two varieties of vegetables as a result of heat processing.

In view of the widespread prevalence of vitamin A deficiency, it is important to understand the extent of bioaccessibility of its precursor, β-carotene from plant foods, which are the main sources of this provitamin, especially in India. While *in vivo* methods of determination of the bioaccessibility of β-carotene are tedious and time consuming, *in vitro* methods offer the advantages of being simpler, more economical and less time consuming. Such methods can be employed for the routine screening of several diverse plant foods for bioaccessibility of β-carotene.

Garrett *et al*. [14] have recently demonstrated that the *in vitro* digestion model , which was earlier used for determining the bioaccessibility of ß-carotene in highly processed infant foods, is also suitable for minimally processed foods such as stir-fried vegetables (which involved stir-frying in vegetable oil at 177°C for 4 min). This *in vitro* digestion system serves as a simple model for screening the relative bioavailability of carotenoids in various plant foods. This quick procedure may particularly be useful for providing insights about dietary strategies for improving vitamin A status in populations that heavily rely on plant foods as the primary source of this vitamin.

4 Concluding remarks

In this study, the suitability of procedural alternatives in the *in vitro* method that is currently in use was examined. This method involved simulated gastro-intestinal digestion followed by separation of the absorbable β-carotene present in the micellar fraction by ultra-centrifugation. Membrane filtration that was tried as an alternative to ultracentrifugation to separate the micellar fraction was found satisfactory, while the simulated gastrointestinal digestion method of Miller *et al.* [15] involving equilibrium dialysis did not produce satisfactory results. Heat processing of vegetables is known to improve the bioaccessibility of micronutrients, including β-carotene. In this investigation two methods of heat processing namely, pressure-cooking and stir-frying, commonly employed in Indian households were examined for their influence on *in vitro* bioaccessibility of β-carotene from its potential sources. Both methods of heat treatment significantly improved β-carotene bioaccessibility from carrot and amaranth leaves. The higher bioaccessibility was particularly prominent when the vegetables were stir-fried in the presence of oil. This information could be used to evolve dietary strategies to derive this micronutrient maximally.

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