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Bioconversion of Carotenoids in Five Fruits and Vegetables to Vitamin A Measured by Retinol Accumulation in Rat Livers

[1,2]Armando Carrillo-Lopez, [1]Elhadi M. Yahia and [1,2]Gabriela K. Ramirez-Padilla
[1]Faculty of Natural Sciences, Autonomous University of Queretaro,
Juriquilla, Querétaro, Qro., México
[2]Food Science and Technology Postgraduate Program, Faculty of Chemical-Biological Sciences,
Autonomous University of Sinaloa, Culiacán, Sinaloa, México

Abstract: Problem statement: Vitamin A deficiency is one of the most prevalent and major nutritional problems in developing countries, especially in young children. In many countries, a substantial proportion of dietary vitamin A is commonly derived from pro-vitamin A carotenoids obtained from colored fruits and orange or green vegetables. However, the bioavailability of retinol derived from carotenoids from these plant sources is not well known. **Approach:** The present study analyzed β-Carotene and Total Carotenoids (TC) composition of carrots (*Daucus carota*), parsley (*Petroselinum crispum*), Spinach (*Spinacea oleracea*), mangoes (*Mangifera indica*) and papayas (*Carica papaya*) and determined the bioconversion of their carotenoids to vitamin A by monitoring the levels of retinol accumulated in liver and plasma of Wistar rats (*Rattus norvegicus*). Products were freeze-dried, β-Carotene content analyzed by HPLC and TC by Spectrophotometry. **Results:** Carrots presented the highest content of β-carotene followed by parsley with 32.8 and 19.6 mg 100 g^{-1}, respectively. Spinach had the highest content of TC followed by parsley with 60.7 and 56.7 mg 100 g^{-1}, respectively. Four-week-old male Wistar rats received a standard diet as an adaptation period, a diet free of Carotenoids and Vitamin A (CVA-diet) as depletion period and finally a Fruit or Vegetable (FoV) based diet as repletion period. The highest β-carotene bioconversion was for mango and the lowest for parsley, whereas the highest TC bioconversion was for carrots and the lowest for parsley. There were no significant differences in plasma retinol between treatments. **Conclusion/Recommendations:** There was no relation between carotenoids content in FoV-based diet and retinol status in plasma. Furthermore, the employment of a general retinol conversion factor is regarded as not appropriate. So, it is recommended to consider specific conversion factors for groups of horticultural crops, for example, a factor for green leafy vegetables and other factor for fruits or roots.

Key words: *Daucus carota*, *Petroselinum crispum*, *Spinacea oleracea*, *Mangifera indica*, *Carica papaya*

INTRODUCTION

Because of humans lack the ability to synthesize vitamin A, we depend on the dietary intake to meet our physiological needs. Furthermore, if our habitual diet provides too little bioavailable vitamin A, some health problems related to vitamin A-deficiency can be suffered. Vitamin A deficiency is one of the most prevalent and major nutritional problems in developing countries, especially in young children (Failla and Chitchumroonchokchai, 2005). The major sources of vitamin A in the diet are preformed vitamin A, commonly found in foods of animal origin and in pro-vitamin-A carotenoids, found in yellow and orange-fleshed fruit and vegetables and in dark-green leafy vegetables (Harrison, 2005). The latter sources are especially important in developing countries (You *et al*., 2002). Of the approximately 600 carotenoids found in nature, only three are important precursors of vitamin A in humans: α-carotene, β-carotene and β-cryptoxanthin. β-Carotene is the major pro-vitamin A component of most carotenoid-containing foods. Moreover, in order to function physiologically as vitamin A, carotenoids-containing food must be well digested to release the carotenoids from the food matrix. This process is followed by absorption through the intestinal mucosa

Corresponding Author: Elhadi M. Yahia, Faculty of Natural Sciences, Autonomous University of Queretaro, Juriquilla, Querétaro, Qro., México Tel: +52-442-1921200/Ext. 5354 Fax: +52-442-2342951

where the conversion to vitamin A partly takes place. The newly formed vitamin A and the unconverted carotenoids are carried by the blood into the liver and other tissues. In the liver, metabolizable carotenoids are further converted to vitamin A and stored (Zacaria-Rungkat *et al*., 2000). As can be seen, the activity of pro-vitamin-A carotenoids as a vitamin A source is uncertain due to concerns about the bioavailability of the carotenoids from vegetables (Graebner *et al*., 2004). Carotenoids bioavailability is determined by several factors such as carotenoid type, food matrix, interaction with other carotenoids and other food components, nutritional status, aging and infections (Yeum and Russell, 2002). Until recently, dietary conversion factor of 6 µg of β-carotene or 12 µg of other pro-vitamin A carotenoids was regarded as equal to 1 µg retinol (Goswami *et al*., 2003). However, some studies have shown that the bioavailability of carotenoids was not as efficient as it was previously thought (Wall, 2006). In 2001, the US Institute Of Medicine (IOM) revised the bioefficacy of β-carotene in a mixed diet from 1:6-1:12, but studies show that a conversion factor of about 1:21 may be more realistic (De Pee *et al*., 1998). Considering the complexity of vitamin A value from food carotenoids, quantitative analysis of carotenoids alone could lead to misinterpretation of their vitamin A value (Zacaria-Rungkat *et al*., 2000). The present study was undertaken to analyze the carotenoid composition of five fruits and vegetables and to determine their bioconversion to vitamin A, by monitoring the levels of retinol in the liver and plasma of Wistar rats fed with these plant products.

MATERIALS AND METHODS

Five horticultural crops were selected based on their high carotenoids content and pro-vitamin A activity. Carrot (*Daucus carota* cv. Bangor), parsley (*Petroselinum crispum* cv. Italian Dark Green), spinach *(Spinacea oleracea* cv. Imperial Spring), mango (*Mangifera indica* cv. Ataulfo) and papaya (*Carica papaya* cv. Maradol). All products were obtained from local market. Thirty 4-week-old male Wistar rats (*Rattus norvegicus*) for the in vivo analysis were obtained from the Medical Faculty at the autonomous university of Queretaro. The fruit and vegetable were selected based on skin color, uniformity of ripeness (fully-ripe fruits), good appearance and freedom of damage. Produce were washed and fresh samples of carrots, parsley, spinach, mangoes and papayas were partially characterized by evaluating their color, total soluble solids and moisture content. Edible tissue was obtained by removing rind, seed or culls. The tissue was cut in pieces of 5 mm (mango, papaya and carrot) or in smaller pieces (spinach and parsley). Then, it was frozen in liquid nitrogen and kept at -80°C and freeze-dried (freeze-dryer model Freestone 1, Labconco Co., Kansas, USA). Freeze-dried samples were placed in air-tight plastic bottles filled with nitrogen, light protected and stored at 4°C until being analyzed for TC, β-carotene content or used in the bioconversion experiment as treatments.

Carotenoids analysis: Carotenoids extraction was carried out following the method of Soto-Zamora *et al*., (2005) with some modifications. Half a gram of freeze-dried tissue was homogenized for 2 min with 15 mL of extraction solution composed of hexane:Acetone: Ethanol:Toluene (10:7:6:7 v/v/v/v), using an Ultra Turrax homogenizer model T25 IKA Works (Labortechnik Inc., Willmington, USA). Then 1 mL 40% KOH in methanol was added, vortexed and stirred for 16 h at 20 RPM at 20°C in light-protected tubes. Fifteen mL of hexane was added and vortexed for 1 min and 15 mL of 10% $NaSO_4$ were added and stirred for 1 min. After 1h-incubation period a phase separation was observed and the upper phase was taken as the extract. Total carotenoids quantification was done measuring the extract absorbance at 450 nm using a DU-65 Beckman spectrophotometer (Fullertone, USA). A calibration curve was done using β-carotene dissolved in hexane as blank. For β-carotene quantification the extract was filtered through 0.45 µm Millipore and placed in injection vials. A Hewlett Packard 1100 HPLC equipment (Agilent Technology, Palo Alto, USA) with diode-array UV-VIS detector was used. A 3.5 µm C18 4.6×150 mm column (Waters, Milford, USA) at 25°C was used. As a mobile phase, acetonitrile-dichloromethane-methanol (82:8:10) at 2 mL min^{-1} flow rate was used. Detection was done using a diode-array detector at 454 nm and 15 min of running time.

Plasma and liver sample preparation: Rats were sacrificed by cervical dislocation and blood was withdrawn from the heart using a syringe and needle containing 10% EDTA. Blood was centrifuged at 10,000 g for 5 min then plasma was separated and kept at -80°C. The livers were excised, washed with 0.9% saline solution, wiped with study towel, weighed and stored at -80°C until assays.

Plasma and liver retinol analysis: Plasma and liver retinol was analyzed by HPLC according to Hosotani and Kitagawa (2003). Retinol extraction from plasma was carried out by mixing 0.15 mL of plasma with 0.35 mL water, 0.5 mL 3% sodium ascorbate and 2 mL ethanol containing 0.135% BHT. The mixture was

vortexed for 20 sec and incubated at 70°C for 5 min. A saponification step was done with 1 mL 10 M KOH at 70°C for 30 min and cooled. Four mL of hexane containing 0.025% BHT was added as extraction solvent. Samples were vigorously vortexed during 10 min and centrifuged at 500×g for 10 min in a Hermle Z 323 K centrifuge (Labortechnik, Wehinghen, Germany). The hexane was recovered. The washing process with the extraction solvent was done three times. Hexane extracts were mixed and dried at 40°C in Rotavapor BUCHI R-205 (Labortechnik, Flawil, Swiss). The residue was redissolved in 500 µL mobile phase, filtered through 0.45 µm Millipore and 10 µL were injected to the HPLC equipment. Retinol extraction from liver was carried out by homogenizing 1 g liver with 10 mL HPLC-grade water in Ultra Turrax T25 IKA Works homogenizer (Labortechnik, Inc., Willmington, USA) at 13,500 RPM during 5 min. 0.3 mL of the homogenate was mixed with 0.7 mL water, 0.5 mL 25% sodium ascorbate, 2 mL methanol and vortexed for 20 sec. After incubation at 70°C for 5 min, the sample was subjected to saponification with 1 mL 10 M KOH at 70°C for 30 min and cooled. About 4 mL of hexane were added as extraction solvent, vortexed vigorously for 10 min and centrifuged at 500×g for 10 min in a Hermle Z 323 K centrifuge (Labortechnik, Wehinghen, Germany). The hexane was recovered. The washing process with extraction solvent was done four times. The n-hexane extracts were mixed and evaporated until dryness at 40°C in a Rotavapor BUCHI R-205 (Labortechnik, Flawil, Swiss). The residue was redissolved in 500 µL of mobile phase, filtered through 0.45 µm Millipore and 10 µL injected into the HPLC equipment. A 3.5 µm Simmetry C18 (4.6×150 mm) column at 30°C was used with acetonitrile-dichloromethane-methanol-1-octanol (90: 15:10:0.1) at 1 mL min^{-1} flow rate. A diode-array detector at 325 nm was used. A calibration curve using retinol dissolved in methanol was done.

Animal and diet handling: For the biological experiment, 4 week-old male Wistar rats were used. Rats received the standard diet NIH-31:18-4 (Zeigler Bros. Inc. Gardners, USA) for two weeks as an adaptation period, followed by a CVA-free diet (AIN-76A, prepared according to Dyets Inc., 2004) for 12 weeks as a depletion period. Then, rats were divided in groups and received for 14 days, the different treatments (each containing approximately 100 µg β-carotene) in addition to CVA-free diet as a control to complete 15 g as total food weight (repletion period). Rats were weighed weekly during the whole biological experiment.

Total Liver Retinol Accumulation (TLRA): This was calculated from the difference of the retinol content contained in liver at the initiation of the repletion period (IRC) and the retinol content after the repletion period.

Retinol accumulation factor: The bioconversion was based on the "Retinol Accumulation Factor" (RAF), which was calculated by dividing the β-carotene or TC intake by the total retinol accumulated in the liver to obtain RAF_{β} and RAF_{TC}, respectively.

Statistical analysis: Data were analyzed by means of analysis of variance and the Fisher test ($\alpha = 0.05$) for comparison of means. Results were presented as means of three repetitions with 3 replicates, unless otherwise stated.

RESULTS

Fruit and vegetables characterization: Physical and chemical characteristics of products were determined at the beginning of the experiment to establish their quality and ripeness stage. Moisture content, total soluble solids contents, internal and external color, TC and β-carotene contents of the five products can be seen in Table 1.

Table 1: Moisture content, Total Soluble Solids (TSS), internal and external color, total carotenoids and β-carotene content in five fruit and vegetables

	Spinach	Mango	Papaya	Parsley	Carrot
Weight (g)[a]	3.16±1.53	286.00±48.1	1.997±409	0.122±0.04	50.60±11.2
Moisture (%)	92.21	78.19	88.370	87.420	88.06
TSS (°Brix)[b]	6.44±0.27	16.93±0.29	10.960±1.20	6.9200±0.14	8.89±0.20
Internal color[+]					
Chroma	19.00±1.6	61.80±2.5	36.500±1.5	20.000±2	45.20±3.3
°Hue	86.00±2.7	71.40±2.4	58.700±3.3	89.700±2.1	45.80±2
External color[++]					
Chroma	15.19±2.1	51.20±2	35.300±5.4	18.000±2	NM
°Hue	80.02±5.8	68.00±2.7	43.500±6.2	88.800±3.3	NM
Total carotenoids[c]	60.69±6.36	5.51±0.39	13.930±0.21	56.650±0.76	40.88±3.80
β-carotene[c]	19.13±0.58	2.56±0.23	1.150±0.014	19.590±0.55	32.78±2.1

[a]: Average weight ± Standard Deviation (SD) of 20 spinach, 8 mangoes, 20 parsley leaves, 4 papayas and 20 carrots [b]: Data are means of 3 repetitions ± SD :with 3 replicates [c]: mg $100g^{-1}$ freeze-dried tissue. TC: Total Carotenoids for spinach and parsley, readings were taken on face ($^{+}$) and reverse ($^{++}$) leaf sides

Table 2: β-Carotene and carotenoids quantities consumed by rats from five fruits and vegetables during repletion period and Total Liver Retinol Accumulation (TLRA)

Treatments	β-Carotene (µg)	Total carotenoids (µg)	TLRA (µg/liver)
Spinach	1376.61±21.10	4367.95±66.96	114.34±13.87
Mango	1220.83±84.99	2629.16±183.03	236.37±6.90
Papaya	1189.10±24.31	14331.90±293.04	252.12±35.66
Parsley	1295.36±18.20	3744.88±52.64	69.14±54.94
Carrot	1405.28±5.32	1752.60±6.64	221.58±15.08

*: Data are means ± standard deviation

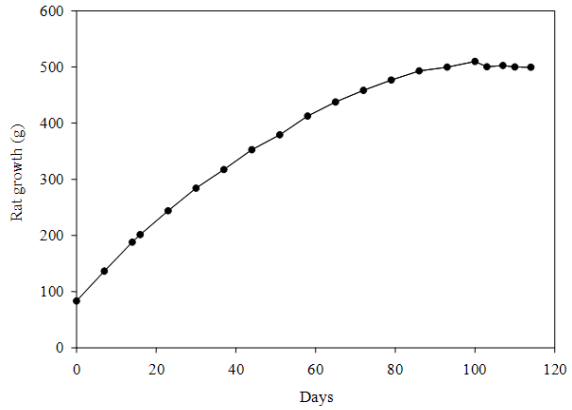

Fig. 1: Growth curve of male "Wistar" rats during the 115 day experimental

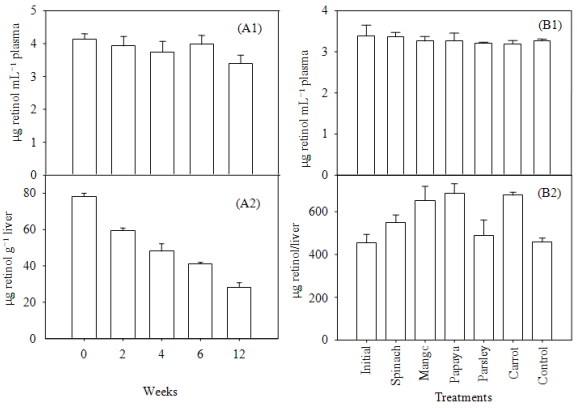

Fig. 2: Retinol depletion in plasma (A1) and liver (A2) and retinol accumulation in plasma (B1) and liver (B2) of male "Wistar" rats after treatments. Data are presented as means and vertical bars indicate standard deviations

β-Carotene and TC analysis: From the analyzed products, carrot presented the highest content of β-carotene followed by parsley, spinach, mango and papaya, with 32.8, 19.6, 19.1, 2.6 and 1.2 mg 100 g^{-1} Freeze-Dried Tissue (FDT), respectively. Spinach had the highest content of TC followed by parsley, carrot, papaya and mango, with 60.7, 56.7, 40.9, 13.9 and 5.5 mg 100 g^{-1} FDT, respectively.

Animal growth: Figure 1 shows the growth of the rats during the whole experimental period. The adaptation period comprised from 0-16 day, in which rats were fed with standard NIH-31 diet (Zeigler), followed by the depletion period from 17-100 day where the rats were fed with CVA-free diet and finally the repletion period from day 101 to day 115, where the rats were fed the different treatments. Rat growth was not affected by treatments. A rapid growth was seen during the first 91 days (13 weeks) with constant weight thereafter. Rat weights stayed unchanged during the last 1.5 weeks of the depletion period and during the 2 weeks of the repletion period. Rats began to lose hair noticeably after 4 weeks of depletion period and, after 6 weeks most rats began to present reddish exudates on their eyes, a symptom of xerophthalmia.

Plasma and liver retinol depletion and accumulation: Figure 2 shows the retinol depletion period (CVA-free diet for 12 weeks). Retinol concentration significantly decreased from 78.16-28.16 µg retinol g^{-1} liver (64% depleted), while in plasma the depletion was from 4.13-3.39 µg retinol mL^{-1} during the same period (18% depleted).

The IRC was 454.6 µg retinol/liver and after FoV-based diet intake for 14 days, retinol content in liver significantly increased, except for parsley treatment and for the negative control (CVA-free diet). The highest retinol-in-liver value was observed for papaya (684 µg retinol/liver), followed by carrot (676), mango (653) and spinach (550) (Fig. 2-B2). The lowest values were shown for parsley (489 µg retinol/liver) and for the negative control (457 µg retinol/liver). As expected, there was no difference between IRC and the negative control. On the other hand, after FoV-based diet intake, there were no significant changes in the retinol content in plasma. Table 2 shows the β-carotene and TC quantities consumed from FoV-diets and the retinol accumulated at the end of the repletion period.

Table 3: Retinol Accumulation Factor (RAF) for β-carotene and for total carotenoids

Treatments	$RAF_{\beta C}$*	RAF_{TC}*
Spinach	1/12.2	1/38.7
Mango	1/5.20	1/11.3
Papaya	1/5.9 0	1/70.5
Parsley	1/27.2	1/78.7
Carrot	1/6.40	1/7.90

RAF is the reciprocal value of total β-carotene or total carotenoids content consumed divided by the total liver retinol accumulation

Papaya exhibited the highest accumulation in liver (254 µg retinol/liver) followed by mango (236.4 µg retinol/liver), carrot (221.6 µg retinol/liver) spinach (114.34 µg retinol/liver) and parsley (69.14 µg retinol/liver). Our results showed that carotenoids bioconversion is lower in green-leafy vegetables (spinach and parsley) compared to fruits (papaya and mango). Machado de Almeida *et al*. (2007) reported that carotenoids bioavalability is lower in leafy vegetables than in fruits or synthetic forms of these compounds.

Retinol accumulation factor: Based on β-carotene or TC intake from FoV diets and retinol repletion in liver, RAF_{β} values were 1/5.2 for mango, 1/5.9 for papaya, 1/6.4 for carrot, 1/12.2 for spinach and 1/27.2 for parsley. RAF_{TC} values were 1/7.9 for carrots, 1/11.3 for mangoes, 1/38.7 for spinach, 1/70.5 for papaya and 1/78.7 for parsley (Table 3). There were no significant differences in plasma retinol between treatments, as can be seen in Fig. 2 (B1).

DISCUSSION

In the present study, we measured the bioconversion of TC and β-Carotene to retinol from fresh fruits and vegetables in rats. The bioconversion analysis of TC to retinol consisted in the depletion of liver retinol and a subsequent repletion with FoV diet. The bioconversion rate was estimated by calculating the retinol increment in plasma and the retinol accumulation in liver in relation to the consumed quantities of TC and β-carotene from FoV diet. This approach was also used by Zacaria-Rungkat *et al*. (2000). We administered from every treatment a quantity of sample equivalent to 100 µg of β-carotene per rat every day and to complete 15 g portions by adding CVA-free diet. Rats did not consume the whole daily portions, but the consumed quantity of β-carotene and TC was calculated and the total quantity administered per treatment during the repletion period was known (Table 2). While liver retinol decreased during the depletion period (64 % of depletion), plasma retinol was reduced only slightly (18% of depletion). Furthermore, while liver retinol significantly increased during the repletion period (except for parsley), plasma retinol did not increase during this period due to any treatment. These results are in agreement with Thurnham and Northrop-Clewes (1999), who suggested that there is no relation between retinol content in plasma and diet, unless the liver retinol pool is depleted noticeably. It seems that a homeostatic process is involved in maintaining the retinol levels in plasma, despite the retinol depletion in liver, as suggested by Zacaria-Rungkat *et al*. (2000). In addition, when there are adequate retinol levels in serum, it has been observed that the carotene-to-retinol bioconversion process is inhibited (Takyi, 2001).

Although green leafy vegetables (spinach and parsley) showed the highest TC and the highest β-carotene content, they exhibited the least bioconversion of β-carotene to retinol as can be seen in Table 3, showing the $RAF_{-\beta}$ to be 1/12.2 for spinach and 1/27.2 for parsley, which means that 12.2 µg of β-carotene intake from spinach results in 1 µg liver retinol or 27.2 µg of β-carotene intake from parsley results in 1 µg retinol in liver. Green leafy vegetables normally possess high-fiber content, which has been reported to interfere with the bioavailability of carotenoids (De Pee and West, 1996; Parker, 1997). Therefore, although green leafy vegetables contain higher quantities of pro-vitamin A carotenoids compared to fruits, the latter possess more bioavailability of their pro-vitamin A carotenoids and therefore most probably more acceptability by humans (McLaren and Frigg, 1999). Although mango and papaya exhibited the lowest TC and β-carotene among the tested products, both of these tropical fruits showed the highest bioconversion of β-carotene to retinol (RAF_{β}) as shown in Table 3. However, in the case of papaya, the TC bioconversion to retinol was quite low with a RAF_{TC} value 1/70.5, suggesting that most of TC in papaya are not pro-vitamin A carotenoids. According to our calculations, β-carotene in papaya accounted for 8% of TC. Carrot, from what carotenoids derived its name, is acknowledged as a pro-vitamin A rich source (McLaren and Frigg, 1999). In our study, carrot had the highest β-carotene content, a high content of TC, a high bioconversion rate of β-carotene to retinol (RAF_{β} equals to 1/6.4) and the highest bioconversion rate of TC to retinol (RAF_{TC} equals to 1/7.9). According to our results, 7.9 µg of TC from carrot resulted in 1 µg of retinol in rat liver, while 6.4 µg of β-carotene from carrot resulted in 1 µg of retinol in rat liver. Zacaria-Rungkat *et al*. (2000), reported a RAF_{β} of 1/12 and a RAF_{TC} of 1/22 for boiled carrot. Besides, spinach,

parsley showed the highest quantity of TC from which about 35% was β-carotene. However, these green-leafy vegetables also showed the least retinol accumulation in rat liver as a result of the poor bioconversion of pro-vitamin A carotenoids to retinol (Table 2). From these results, fresh parsley cannot be recommended as a rich source of vitamin A. However, some heat treatments such as immersion for 1-3 min in boiling water, vapor or hot air have shown to increase the bioavailability of carotenoids without reducing the carotene content. This might be due to the fact that these treatments release carotenoids from carotenoids-protein complexes, therefore becoming more bioavailable (Boileau *et al*., 1999). Actually, there are many factors that affect carotenoids bioavailability, including type of carotenoid, unions at molecular level, amount of intake, food matrix, factors related to the individual, problems of absorption, nutritional state, genetics and interactions among these variables (Castenmiller *et al*., 1999; Rock *et al*., 1998; Van Het Hof *et al*., 2000). In short, the bioavailability of carotenoids from food as sources of vitamin A is affected by endogenous and exogenous factors that are complex, varied and difficult to predict. Other conditions such as type of fruit or vegetable, type of soil of cultivation, climate and storage conditions can also have significant effects. Therefore, one must be careful to extrapolate the results of a product to another, or even with the same type of product grown in different seasons of the year, because the variations can influence the type and size of carotenoid-protein complexes and therefore the amount of vitamin A (Takyi, 2001). On the other hand, it is important to know the best way to prepare and store food containing carotenoids in order to maintain and even improve its bioavailability, especially in populations at risk (Olson, 1999).

CONCLUSION

There was no relation between carotenoids content in FoV-based diet and retinol status in plasma. Spinach and parsley (green-leafy vegetables) and carrot (root), showed higher carotenoids content compared to papaya and mango (fruits). However, carotenoids from mangoes, papaya and carrots showed more bioavailability, producing a higher bioconversion to retinol, while green-leafy vegetables were less effective in accumulating liver retinol. The observed differences in bioconversion of carotenoids to vitamin A, suggest that the type of plant food as pro-vitamin a source is important to take into account when it comes to recommending them as retinol sources. Furthermore, the use of a general retinol conversion factor is not appropriate. From our results, it seems that there is a need for specific conversion factors for the different groups of horticultural commodities; for example, a factor for green leafy vegetables and other factor for fruits or roots.

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