Occurrence of *Cis* Isomers of Provitamins A in Brazilian Vegetables

Helena T. Godoy* and Delia B. Rodriguez-Amaya

Departamento de Ciência de Alimentos, Faculdade de Engenharia de Alimentos, Universidade Estadual de Campinas, C.P. 6121, 13081-970 São Paulo, Brazil

Because *cis* isomerization lowers provitamin A activity, concern has been raised over the necessity of separating the provitamin A isomers in determining the vitamin A values of foods. Sixteen different raw vegetables and eight cooked vegetables (total of 123 samples) were analyzed to appraise the occurrence of *cis* isomers. The raw vegetables analyzed, except tomato and carrot, had *cis* isomers (trace to 3.4 μg/g 13-*cis*-β-carotene and trace to 4.1 μg/g 9-*cis*-β-carotene). The *trans*-β-carotene range was 1.1−38.4 μg/g. Appreciable amounts of α-carotene were found only in carrot (16.5−19.0 μg/g *trans*-α-carotene) and in a cultivar of squash (0.3 μg/g 13-*cis*-α-carotene, 17.1 μg/g *trans*-α-carotene, and 0.1 μg/g 9-*cis*-α-carotene). The vitamin A values were calculated with and without isomer separation, using currently accepted biological activities, showing overestimations of 10−22% when the isomers were not separated. *Cis* isomers were found in all samples of cooked vegetables analyzed; the overestimations ranged from 5 to 20%. Isomer separation may be necessary for some vegetables, but the biopotency of the isomers has to be reevaluated.

Keywords: *Provitamin A determination; carotenoid geometric isomers; vegetables*

INTRODUCTION

With the advent of high-performance liquid chromatography (HPLC), the necessity of separating the less potent *cis* isomers from the *trans*-provitamins A in determining the vitamin A values (activities) of plant foods has once again been raised. Even with this powerful technique, the separation is not easily accomplished. After several evaluations, two columns appeared to be the most efficient for resolving the isomers of β-carotene: Vydac 201 TP C_{18} (Bushway, 1985; Quackenbush and Smallidge, 1986; Craft et al., 1990; Pettersson and Jonsson, 1990; O'Neil et al., 1991; Chen and Chen, 1994) and laboratory-packed $Ca(OH)_2$ (Tsukida et al., 1982; Chandler and Schwartz, 1987; O'Neil et al., 1991; Schmitz et al., 1995). When α- and β-carotene isomers were separated simultaneously, the late eluting *cis* isomers of α-carotene overlapped with the early eluting β-carotene isomers (Pettersson and Jonsson, 1990; Chen and Chen, 1994).

A polymeric C_{30} stationary phase was recently introduced for improved resolution of carotenoids (Sander et al., 1994) and was successfully used for the separation of *cis* and *trans* carotenoid standards and those extracted from biological sources (Emenhiser et al., 1995, 1996a,b; Lessin et al., 1997). Emenhiser et al. (1996b) separated geometric isomers of α-carotene and β-carotene of thermally processed carrots, and Lessin et al. (1997) separated the isomers of β-carotene, α-carotene, and β-cryptoxanthin of several fruits and vegetables.

Even if a good separation of the isomers of different provitamins were achieved, determination of the absolute concentrations is problematic. The calibration techniques require reliable carotenoid standards, which are difficult to obtain and maintain. Of the *trans*-provitamins, only α- and β-carotenes are easily acquired commercially, and the purity of commercial β-carotene preparations has been shown to vary markedly (Quackenbush and Smallidge, 1986; Craft et al., 1990). Obtention of *cis* isomer standards is even more difficult.

The isomers can be separated and quantified individually by open column chromatography (classical column chromatography) and visible absorption spectrometry, but two columns are needed (Bickoff et al., 1949; Sweeney and Marsh, 1970). According to Tsukida (1992), this traditional method is the most effective and practical way of dealing with isomeric mixtures of carotenoid in quantity. The provitamins are first isolated on an MgO:HyfloSupercel column, and each provitamin is rechromatographed on a $Ca(OH)_2$ column for isomer separation. Reproducibility and efficiency of the chromatographic separation depend heavily on the analyst's skill and experience.

An additional problem is the possibility of forming *cis* isomers during analysis. This isomerization, catalyzed by light, heat, acids, and active surfaces, is considered the most common artifact problem in carotenoid analysis (Liaaen-Jensen, 1990). This could happen, for example, on hot saponification (Kimura et al., 1990) and on standing with chlorinated solvents, such as chloroform and methylene chloride (Pesek et al., 1990), even in the dark. In addition, Khachik et al. (1988) observed peak splitting when *trans* carotenoids were injected in methylene chloride, chloroform, tetrahydrofuran, benzene, or toluene with a C_{18} column and a mixture of methanol, acetonitrile, methylene chloride, and hexane as mobile phase. These HPLC peak artifacts could be misidentified as *cis* isomers. Any report on the presence of *cis* isomers should therefore guarantee that they are natural constituents and not analytical artifacts.

An appraisal of the natural occurrence of *cis* isomeric provitamins is needed before the difficult and error-prone *cis* and *trans* isomer separation is required in official methods and in generating data for food composition tables. Using HPLC, *cis* isomers of β-carotene had been found in some fresh and processed foods

* Author to whom correspondence should be addressed.

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Table 1. *Cis* and *Trans* Isomer Concentrations of Provitamins A in Fresh and Cooked Brazilian Vegetables

vegetable, cultivar	domestic preparation	no. of samples analyzed[a]	provitamin A	mean concn (μg/g of edible portion)
broccoli	raw	4	13-*cis*-β-carotene	1.2 ± 0.4
			trans-β-carotene	18.1 ± 1.3
			9-*cis*-β-carotene	1.5 ± 0.6
	boiled	4	13-*cis*-β-carotene	0.6 ± 0.2
			trans-β-carotene	14.9 ± 1.1
			9-*cis*-β-carotene	0.8 ± 0.5
carrot				
Imperador	raw	5	*trans*-α-carotene	19.0 ± 0.8
			trans-β-carotene	38.4 ± 4.1
	boiled	4	*trans*-α-carotene	16.3 ± 0.4
			13-*cis*-β-carotene	0.2 ± 0.1
			trans-β-carotene	36.1 ± 2.0
			9-*cis*-β-carotene	0.1 ± 0.1
Nantes	raw	5	13-*cis*-α-carotene	tr[b] in three samples
			trans-α-carotene	16.5 ± 2.3
			13-*cis*-β-carotene	tr[b] in two samples
			trans-β-carotene	33.0 ± 2.4
	boiled	4	13-*cis*-α-carotene	0.2 ± 0.1
			trans-α-carotene	13.4 ± 1.2
			13-*cis*-β-carotene	0.2 ± 0.1
			trans-β-carotene	30.3 ± 1.6
			9-*cis*-β-carotene	0.1 ± 0.1
green beans				
Macarrão	raw	5	*trans*-α-carotene	0.2 ± 0.1
			13-*cis*-β-carotene	0.2 ± 0.1
			trans-β-carotene	1.2 ± 0.2
			9-*cis*-β-carotene	0.3 ± 0.1
	boiled	5	trans-α-carotene	0.2 ± 0.1
			13-*cis*-β-carotene	0.2 ± 0.2
			trans-β-carotene	1.0 ± 0.2
			9-*cis*-β-carotene	0.1 ± 0.1
	stir-fried	5	13-*cis*-β-carotene	0.2 ± 0.1
			trans-β-carotene	0.8 ± 0.2
			9-*cis*-β-carotene	0.1 ± 0.1
green corn	raw	5	13-*cis*-β-carotene	0.4 ± 0.2
			trans-β-carotene	1.1 ± 0.2
			9-*cis*-β-carotene	0.2 ± 0.1
			trans-β-cryptoxanthin	1.0 ± 0.4
Indian eggplant	raw	5	13-*cis*-β-carotene	tr[b] in two samples
			trans-β-carotene	1.4 ± 0.4
			9-*cis*-β-carotene	0.1 ± 0.1
	boiled	5	13-*cis*-β-carotene	0.2 ± 0.1
			trans-β-carotene	1.4 ± 0.3
			9-*cis*-β-carotene	0.2 ± 0.1
kale	raw	5	13-*cis*-β-carotene	3.4 ± 2.1
			trans-β-carotene	25.8 ± 4.4
			9-*cis*-β-carotene	4.1 ± 2.8
okra	raw	5	13-*cis*-β-carotene	0.2 ± 0.1
			trans-β-carotene	2.7 ± 0.6
			9-*cis*-β-carotene	0.2 ± 0.1
	boiled	5	13-*cis*-β-carotene	0.1 ± 0.1
			trans-β-carotene	1.8 ± 0.6
			9-*cis*-β-carotene	0.2 ± 0.2
pepper				
green	raw	4	13-*cis*-β-carotene	0.2 ± 0.1
			trans-β-carotene	2.1 ± 0.4
			9-*cis*-β-carotene	0.2 ± 0.2
yellow	raw	4	*trans*-α-carotene	0.5 ± 0.2
			13-*cis*-β-carotene	0.1 ± 0.1
			trans-β-carotene	1.5 ± 0.3
			9-*cis*-β-carotene	0.3 ± 0.2
			trans-α-cryptoxanthin	0.8 ± 0.5
red	raw	4	13-*cis*-β-carotene	0.4 ± 0.2
			trans-β-carotene	3.8 ± 0.7
			9-*cis*-β-carotene	0.2 ± 0.2
spinach	raw	5	13-*cis*-β-carotene	2.3 ± 1.2
			trans-β-carotene	25.0 ± 4.4
			9-cis-β-carotene	2.7 ± 0.8
	boiled	5	13-*cis*-β-carotene	1.2 ± 0.2
			trans-β-carotene	20.5 ± 2.0
			9-cis-β-carotene	1.8 ± 0.7
squash				
Menina Verde	raw	5	13-*cis*-α-carotene	0.3 ± 0.2
			trans-α-carotene	17.1 ± 2.3
			9-*cis*-α-carotene	0.1 ± 0.1

Table 1 (Continued)

vegetable, cultivar	domestic preparation	no. of samples analyzed[a]	provitamin A	mean concn (μg/g of edible portion)
			13-*cis*-β-carotene	0.2 ± 0.1
			trans-β-carotene	23.6 ± 3.2
			9-*cis*-β-carotene	0.3 ± 0.2
			trans-α-cryptoxanthin	0.7 ± 0.4
	boiled	5	13-*cis*-α-carotene	0.8 ± 0.3
			trans-α-carotene	15.6 ± 2.1
			9-*cis*-α-carotene	0.3 ± 0.2
			13-*cis*-β-carotene	1.0 ± 0.4
			trans-β-carotene	21.1 ± 2.5
			9-*cis*-β-carotene	0.4 ± 0.2
			trans-α-cryptoxanthin	0.6 ± 0.2
	stir-fried	5	*trans*-α-carotene	18.0 ± 3.1
			trans-β-carotene	20.7 ± 1.8
			9-*cis*-β-carotene	0.7 ± 0.2
			trans-α-cryptoxanthin	0.7 ± 0.1
tomato				
Santa Cruz	raw	5	*trans*-β-carotene	5.1 ± 0.4
			trans-γ-carotene	0.7 ± 0.3
Marglobe	raw	5	*trans*-β-carotene	6.2 ± 0.3
			trans-γ-carotene	0.6 ± 0.2
watercress	raw	5	13-*cis*-β-carotene	1.8 ± 1.1
			trans-β-carotene	23.3 ± 2.8
			9-cis-β-carotene	2.2 ± 1.2

[a] Each sample lot was analyzed in duplicate. [b] tr, trace.

(Bushway, 1985; Chandler and Schwartz, 1987; Quackenbush, 1987; Pettersson and Jonsson, 1990; O'Neil et al., 1991; Saleh and Tan, 1991; Chen and Chen, 1994). Lessin et al. (1997) quantified isomers of β-carotene, α-carotene, and β-cryptoxanthin in fresh and processed fruits and vegetables. In these studies, one or two sample lots of each food were analyzed, the concentrations in weights per gram of sample were presented only in two works (O'Neil et al., 1991; Lessin et al., 1997), and the results were not converted to retinol equivalents. In 12 types of fresh fruits, for which five samples lots were analyzed for each fruit, only mamey, nectarine, peach, and *Mauritia vinifera* presented measurable amounts of provitamin A *cis* isomers (Godoy and Rodriguez-Amaya, 1994). Even with these fruits, the overestimations incurred from nonseparation of the isomers were negligible once the results were transformed to retinol equivalents. In tomato, β-carotene *cis* isomers were not detected in 10 fresh sample lots but were found in various concentrations in 52 samples of commercially processed products, reaching high levels in some samples (Rodriguez-Amaya and Tavares, 1992). For these products, therefore, appreciable overestimations of the vitamin A values occurred when the isomers were not separated.

From the reported results, *cis* isomers seemed to be more prevalent in vegetables. Their presence, especially in photosynthetic tissues, may be related to the physiological role of carotenoids (Britton, 1991). In the present work a more extensive investigation of the *cis* isomer distribution in vegetables was undertaken.

MATERIALS AND METHODS

Sample Collection and Preparation. The samples were purchased from supermarkets and groceries in Campinas, Brazil. Each sample lot consisted of five carrots; five ears of corn; three peppers; 200 g each of broccoli (*Brassica oleracea*) and the leafy vegetables kale (*Brassica oleracea* var. acephala), spinach (*Spinacea oleracea*), and watercress (*Nastrutium officinale*); 500 g each of tomato (*Lycopersicon esculentum*), okra (*Hibiscus esculentus*), Indian eggplant (*Solanum melongena* var. Brisyal), and green beans (*Phaseolus vulgaris*); or a single squash (*Curcubita moschata*). Carrot, pepper, leafy vegetables, green beans, and broccoli were cut into pieces before homogenization in a Waring blender. Corn was removed from the cob, and tomato was directly homogenized. The squash was quartered, and opposite sections were taken and homogenized. Samples of 5−20 g, depending on the carotenoid content, were taken for analysis.

The samples were analyzed immediately after purchase, taking all necessary precautions to avoid isomerization and degradation during analysis, such as rapid analysis, protection from light and high temperature, use of BHT during homogenization to prevent oxidation, exclusion of saponification, and use of distilled, reagent grade, peroxide-free, and acid-free solvents.

Some vegetables were cooked as is commonly done in Brazilian homes. After cutting, the vegetable pieces were immersed in boiling water or stir-fried in a minimum amount of oil until just done as judged by texture. Boiling took 3 min for India eggplant and spinach and 5 min for broccoli, green beans, and okra. Carrot and squash required 8 and 10 min of boiling, respectively. Stir-frying of green beans and squash took 8 and 10 min, respectively.

Carotenoid Determination and Calculation of the Vitamin A Value. Extraction of the carotenoids and separation of the provitamins from each other and from interfering non-provitamin A carotenoids on an MgO:HyfloSupercel column were done as described previously (Rodriguez-Amaya et al., 1988). The provitamin A fractions were rechromatographed in $Ca(OH)_2$ columns to separate the *cis* and *trans* isomers (Rodriguez-Amaya and Tavares, 1992; Godoy and Rodriguez-Amaya, 1994). Evaluation of the different steps of the analytical procedure was discussed in the previous papers. Losses in the $Ca(OH)_2$ column were 4% for *trans*-β-carotene and 6% for *trans*-β-cryptoxanthin.

The carotenoids were identified by the combined use of UV−visible absorption spectra, chromatographic behaviour, and chemical reactions, such as iodine-catalyzed isomerization to verify the isomeric form (Rodriguez et al., 1976; Godoy and Rodriguez-Amaya, 1994). Additionally, the major *cis* isomers of β-carotene, eluting before and after *trans*-β-carotene in the $Ca(OH)_2$ column, were shown to be 13-*cis*-β-carotene and 9-*cis*-β-carotene, respectively, on the bases of their 200-MHz ^1H NMR and 50.3-MHz ^{13}C NMR spectra (Tsukida et al., 1981). The separated provitamin A fractions were quantitated spectrophotometrically according to the method of Davies (1976),

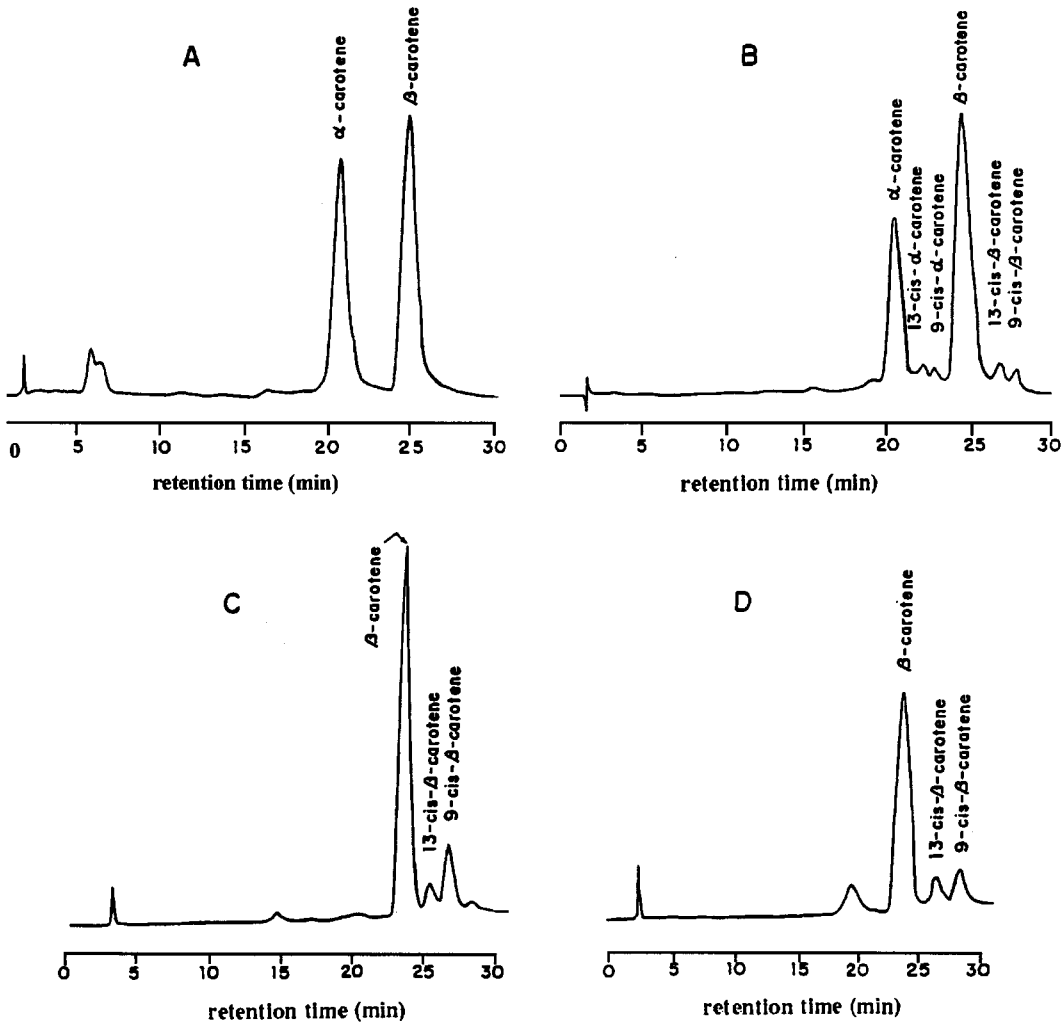

Figure 1. HPLC chromatogram of the total extracts of (A) carrots and (B) squash and β-carotene fraction obtained from MgO column of (C) spinach and (D) green beans.

using $A^{1\%}_{1cm}$ obtained in our laboratory (Godoy and Rodriguez-Amaya, 1994).

The vitamin A values were calculated, using the NAS−NRC (1989) conversion ratio of 6 μg of β-carotene to 1 RE (retinol equivalent (RE) and accepted bioactivities (Zechmeister, 1962; Bauernfeind, 1972) of the different provitamins (13-*cis*-α-carotene, 13%; *trans*-α-carotene, 50%; 9-*cis*-α-carotene, 16%; 13-*cis*-β-carotene, 53%; *trans*-β-carotene 100%; 9-*cis*-β-carotene, 38%; *trans*-β-cryptoxanthin, 57%). The vitamin A values were also calculated using the biopotencies obtained by Sweeney and Marsh (1973) (74% for 13-*cis*-β-carotene and 61% for 9-*cis*-β-carotene).

Confirmation of the Presence of *Cis* Isomers by HPLC. As in the previous study (Godoy and Rodriguez-Amaya, 1994), the presence of isomers was confirmed by HPLC using a Varian liquid chromatograph (Palo Alto, CA). The equipment consisted of a ternary solvent delivery system (model 5010), a Varian UV−vis detector (model 5100), a Rheodyne manual injection switching valve (10 μL sample loop), and an integrator−recorder (model 4400). The isocratic separation was performed on a 250 × 4.6 i.d. mm Vydac 201 TP54 C_{18} (5 μm) column (Hesperia, CA) preceded by a 30 × 4.6 i.d. mm Varian Micropore MCH-120 C_{18} (10 μm) guard column with methanol/water (98:2) as mobile phase. Flow rate was set at 1.5 mL/min and detection at 450 nm. Cochromatography was used to locate the isomers of the provitamins, corroborated by the spectra obtained with a Waters diode array detector (model 994) (Marlborough, MA). 13-*cis*-β-Carotene and 9-*cis*-β-carotene standards were isolated from kale as described previously (Godoy and Rodriguez-Amaya, 1994). These isomers obtained from the vegetables analyzed in the present study, separated by the $Ca(OH)_2$ colunm, were also injected into the liquid chromatograph.

RESULTS AND DISCUSSION

Cis isomers were found, generally at low levels, in all of the fresh vegetables analzed, except carrot cultivar Imperador (5 samples) and tomato (10 samples) (Table 1); a trace of 13-*cis*-β-carotene was noted in some samples of carrot cultivar Nantes. Lessin et al. (1997) did not find *cis* isomers of α- and β-carotene in fresh carrot as well. Only carrot and squash cultivar Menina Verde presented significant amounts of α-carotene; green beans and yellow pepper had trace or low levels. β-Cryptoxanthin in small amount was found in green corn, whereas squash and yellow pepper had α-cryptoxanthin.

The provitamin A composition of some cooked vegetables was also determined (Table 1). All samples, including carrot, had *cis* isomers of β-carotene. This result is understandable because heat treatment of foods has been shown to promote *cis* isomerization (Gortner and Singleton, 1961; Panalaks and Murray, 1970; Sweeney and Marsh, 1971; Lee and Ammerman, 1974; Ogunlesi and Lee, 1979; Chandler and Schwartz, 1988; Chen and Chen, 1994). 13-*cis*-α-Carotene appeared only in the carrot Nantes and squash and 9-*cis*-α-carotene only in squash. Because of thermal isomerization, the

Table 2. Vitamin A Values of Fresh and Cooked Vegetables Calculated with and without Isomer Separation

vegetable, cultivar	domestic preparation	vitamin A value[a] (RE/ 100 g) without isomer separation	with isomer separation *b*	with isomer separation *c*	with isomer separation *d*	overestimation (%) *b*	overestimation (%) *c*	overestimation (%) *d*
broccoli	raw	397	322	332	345	23	19	15
	boiled	298	259	271	276	15	10	8
carrot								
Imperador	raw	798						
	boiled	790	740	772	*e*	7	2	*e*
Nantes	raw	899						
	boiled	686	620	647	*e*	10	6	*e*
green bean								
Macarrão	raw	29	26	27	28	11	7	4
	boiled	24	21	22	24	14	9	0
	stir-fried	19	16	17	18	19	12	5
green corn	raw	41	32	35	38	28	17	8
Indian eggplant	raw	29	24	25	26	20	16	12
	boiled	29	25	26	28	16	12	4
kale								
Manteiga	raw	598	486	510	538	23	17	11
okra	raw	57	48	50	52	19	14	10
	boiled	38	32	34	36	19	12	5
pepper								
green	raw	47	38	40	42	24	17	12
yellow	raw	49	39	42	46	26	17	6
red	raw	87	68	72	75	28	20	16
spinach	raw	538	454	475	482	18	13	12
	boiled	427	364	381	392	17	12	9
squash								
Menina Verde	raw	599	543	568	*e*	10	5	*e*
	boiled	627	502	527	*e*	24	19	*e*
	stir-fried	525	499	520	*e*	5	1	*e*
tomato								
Santa Cruz	raw	91						
Marglobe	raw	104						
watercress	raw	486	418	432	446	16	12	9

[a] Values are means of four or five determinations in duplicate. [b] Calculated according to the bioactivities obtained by Zechemeister's group (1962). [c] Calculated according to the bioactivities obtained by Zechemeister's group (1962) corrected for loss on column. [d] Calculated according to the bioactivities obtained by Sweeney and Marsh (1973), corrected for loss on column. [e] Not calculated; bioactivities of α-carotene isomers were not determined by Sweeney and Marsh (1973).

lower levels of the *cis* provitamins in the cooked vegetables appear surprising at first glance. However, carotenoids, *cis* or *trans*, also undergo degradation when heated. Additionally, some water was absorbed by the boiled sample, thus lowering the amount of the carotenoids on a per gram sample basis.

The occurrence of provitamin A isomers, as shown by the open column determinations, was confirmed by HPLC. Quantitation was not carried out by HPLC, however, because of the difficulty in obtaining enough *cis* isomer standards to construct the calibration curves. Our chromatograms (Figure 1) are comparable with those obtained by other authors with the Vydac column (Quackenbuch, 1987; Pettersson and Jonsson, 1990; O'Neil et al., 1991; Chen and Chen, 1994). *Cis* isomers of α- and *β*-carotene were not found in raw carrot but were detected in squash. 9-*cis* and 13-*cis*-*β*-carotene were encountered in red pepper but not in tomato. The green vegetables analyzed, without exception, had *cis* isomers of *β*-carotene.

The vitamin A values (activities) of the vegetables containing *cis* isomers were calculated with and without isomer separation, using the bioactivities obtained by Zechmeister's group based on rat growth (weight gain) (Zechmeister, 1962). In the fresh vegetables, overestimations of 10−22% were observed when the isomers were not separated (Table 2), overestimations being greater for broccoli, kale, green corn, and pepper. These percentages would be lower (3−14%) if losses on the $Ca(OH)_2$ column and the bioactivities reported by Sweeney and Marsh (1973), based on rat liver reserve of vitamin A, were considered.

The mean overestimation in cooked vegetables (Table 2), using the biopotencies obtained by Zechmeister's group, varied from 5 to 20%, being greater for stir-fried green beans, boiled okra, boiled squash, and boiled spinach. If provitamin losses on the column and the bioactivities obtained by Sweeney and Marsh (1973) were taken into consideration, overestimations would be reduced to 0−8%.

Overestimation of the vitamin A value is more common and higher in vegetables than in fresh fruits. Thus, isomer separation may be important for some vegetables. However, before this difficult and error-prone step is required in official methods of analysis, reevaluation of the biopotencies of the *cis* and *trans* isomers of at least the principal provitamins is needed. Reported biopotencies based on growth (which is not a specific parameter) and vitamin A liver reserve of rats (a questionable model for man) were different. At the moment, considering the natural variation between food samples (shown by the standard deviations in Table 1), the analytical difficulty in determining the *cis* isomers, and the lack of definition of the bioavaibility of the provitamins, the significance of the error associated with the nonseparation of *cis* isomers from the nutritional rather than the analytical standpoint is unclear.

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