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Analytical Methods Section

Importance of *cis*-isomer separation in determining provitamin A in tomato and tomato products

Delia B. Rodriguez-Amaya & Cassia A. Tavares

Departamento de Ciência de Alimentos, Faculdade de Engenharia de Alimentos, Universidade Estadual de Campinas, 13081 Campinas, SP, Brazil

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An attempt was made to estimate the error in the determination of the vitamin A value (activity) when the *cis*- and *trans*-isomers are not separated and quantified individually. In 10 sample lots of fresh tomatoes, *cis*-isomers were not detected. However, in 52 samples of tomato products, varying amounts of *cis*-isomers of β-carotene were found. Utilising the currently employed calculation procedure, overestimations of 8–13% in juice, 10–66% in puree and paste, 15–26% in ketchup were observed when isomer separation was not undertaken. When column loss was taken into consideration, these errors decreased to 4–8%, 6–62% and 11–21%, respectively. Using the biopotencies obtained by Sweeney and Marsh (1970), aside from column loss, the overestimations were 4–8%, 3–52% and 7–15%, respectively. Thus, the error can be considerable, but the magnitude depends on how the calculation is carried out, demonstrating the necessity of establishing the true biopotencies, aside from obtaining more efficient chromatographic separation and accurate quantitation.

INTRODUCTION

Not all carotenoids are provitamins and those which are vary in their biological activity. To determine the vitamin A values of plant foods accurately it is necessary: (1) to separate interfering inactive carotenoids; (2) to separate and quantify the provitamins individually; and (3) to quantify the *cis*- and *trans*-isomers of each provitamin (Rodriguez-Amaya, 1989). The difficulty in carrying out the analysis, however, increases as these requirements are fulfilled. Thus, provitamin A determination has been accomplished with varying degrees of completeness and the vitamin A value (or activity) calculated on the basis of the concentrations of: (1) only β-carotene; (2) α- and β-carotene; (3) α-, β-carotene, β-cryptoxanthin; (4) *cis*- and *trans*-isomers of β-carotene. Rarely are all the different provitamins and in their different isomeric forms, present in the food sample, individually quantified.

Carotenoids occur in nature primarily in the more stable all-*trans* form; however, *cis*-isomers do exist naturally. Since *cis*-isomeric provitamins are less potent, the separate quantitation of the isomers in provitamin A determination has been recommended. The structure–biopotency relationship of the isomeric provitamins A was eloquently discussed by Zechmeister (1949). In terms of the visible absorption spectrum, *cis*-isomerisation of one of the chromophore's bonds results in a hypsochromic shift and hypochromic effect, accompanied by the appearance of a new '*cis*' peak in the ultraviolet region (Vetter *et al.*, 1971; Davies, 1976).

Separation of *cis*-isomers is not an easy task. In open column (also called gravity-flow column) methods, it requires rechromatography of each of the provitamin fractions, obtained from the MgO/Hyflosupercel column in a $Ca(OH)_2$ column (Bickoff *et al.*, 1949) or a $Mg(OH)_2/Ca(OH)_2$ column (Sweeney & Marsh, 1970). Separation efficiency and reproducibility, especially in the second column, depend largely on the analyst's skill and experience. HPLC is potentially an excellent method for the separation and quantification of provitamin A carotenoids and their isomers. Although many methods do not satisfactorily separate isomeric forms, $Ca(OH)_2$ and some C_{18} columns do allow separation of the isomers. However, the problem is complicated

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when their accurate identification and quantification is required.

Testing four commercial C_{18} columns with five solvent systems and normal-phase columns (amino and alumina), Bushway (1985) obtained chromatograms which demonstrated the difficulty in separating different carotenoids and their *cis-trans* isomers. Quackenbush and Smallidge (1986) tested more than 20 C_{18} and C_8 commercial columns and only three C_{18} columns showed some separation of the *cis* and *trans* forms of β-carotene, with Vydac TP-201 being the most effective. Some food samples were analysed and the 13-*cis*- and 9-*cis*-β-carotene levels were given as percentages of the total β-carotene (Quackenbush, 1987). Even with gradient elution the *cis*-isomer, designated tentatively as 15-*cis*-β-carotene, appeared as a shoulder of, and was quantified with, *trans*-β-carotene in Khachik *et al.* (1986, 1988, 1989) and Heinonen *et al.* (1989).

Separation of the isomers of β-carotene was achieved on a laboratory packed $Ca(OH)_2$ column developed with 0·1 or 0·5% acetone in hexane (Tsukida *et al.*, 1982) or with acetone/hexane (3:997, v/v) (Chandler & Schwartz, 1987). The latter system was applied to some food samples but only the relative ratios (in %) were reported. Calculation was based on the absorbance at 463 nm, close to the isobestic point, assuming similar absorptivities and using a single calibration curve (that of *trans*-β-carotene). Thus, although the results showed that the *cis*-isomer levels could be significant, especially in processed products, the different foods could not be compared quantitatively and the vitamin A values could not be calculated.

Also using a $Ca(OH)_2$ column and 0·5–1·0% acetone in hexane as mobile phase, Pettersson & Jonsson (1990) discussed diode array detection of the isomers of α- and β-carotene. The authors noted the difficulties in obtaining fresh and reliable α- and β-carotene standards and in separating very closely related compounds within a short analysis time. Another problem cited was the sensitivity of $Ca(OH)_2$ to water and other polar solvents. Although the emphasis of the paper was isomer separation, the quantitative data (for heat-treated carrot juice) were given in terms of α- and β-carotene, without distinguishing the isomers.

Surprisingly, in none of the HPLC studies were the provitamin A data converted to vitamin A values or activities. Reports of *cis*-isomer presence and levels should also guarantee that they are true constituents of the sample, not artifacts formed during the analysis.

This study was undertaken as an attempt to verify whether an official method for provitamin A should make provision for isomer separation and Food Composition Tables should specify the provitamin A contents up to the isomer level. The error involved when the *cis*-isomers were not separated was quantified. To obtain a better picture, a large number of samples of tomato and tomato products were examined. Studies of the *cis*-isomers have generally only involved a few samples of some foods.

MATERIALS AND METHODS

Materials

Fresh and processed samples of tomato were bought from groceries or supermarkets. For each sample lot, the tomatoes were quartered and opposite sections were homogenised in a Waring blender. Since they had already undergone homogenisation during processing, the processed products (from each container) were simply mixed and samples were taken for analysis. Samples of 60 g each were submitted for analysis.

Carotenoid determination

The steps from extraction to separation in a MgO/Hyflosupercel (1:1, activated) column had been described previously (Rodriguez-Amaya *et al.*, 1988). Briefly this involved extraction with acetone, partition to petroleum ether, concentration in a rotary evaporator at a temperature not exceeding 35°C and chromatographic separation, β-carotene being eluted with 2% acetone and γ-carotene with 8% acetone in petroleum ether. Saponification was not found necessary. The different steps of this procedure had been assessed previously (Rodriguez-Amaya *et al.*, 1988; Kimura *et al.*, 1990). The β-carotene fraction obtained from the MgO/Hyflosupercel column was rechromatographed on a $Ca(OH)_2$ (Mallinkrodt) column. Elution was carried out with petroleum ether for 13-*cis*-β-carotene, 2% ethyl ether for *trans*-β-carotene and 4% ethyl ether in petroleum ether for 9-*cis*-β-carotene.

Confirmation of the identity of β-carotene was based on the visible absorption spectrum and chromatographic behaviour. Iodine catalysed isomerisation was undertaken to verify the isomeric form and quantitation was done spectrophotometrically (Davies, 1976), using $A^{1\%}_{1\,cm}$ of 2592 for *trans*-β-carotene in petroleum ether.

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-mono-*cis*- and 9-mono-*cis*-β-carotene, respectively, on the bases of their 200 MHz ^{1}H- and 50·3 MHz ^{13}C-NMR spectra (Tsukida *et al.*, 1981). For the calculation of the β-carotene *cis*-isomer concentrations, the $A^{1\%}_{1\,cm}$ obtained by Sweeney and Marsh (1970) were used (2360 for 13-*cis*-β-carotene and 1930 for 9-*cis*-β-carotene).

RESULTS AND DISCUSSION

In ten sample lots of fresh tomatoes, containing 506 ± 108 μg per 100 g β-carotene the *cis*-isomers were not

detected. Chandler and Schwartz (1987), using HPLC, also reported no *cis*-isomers in the single fresh tomato sample analysed. The fresh samples also contained 70 ± 20 μg per 100 g of γ-carotene, but this provitamin was not encountered in the processed products.

The *cis*-isomers appeared in 52 processed tomato samples (two brands) (Tables 1 and 2). Thus, tomato follows the general trend that *cis*-isomers are formed or increased during processing. The results also agreed with the observation of Sweeney and Marsh (1970) that the principal isomer formed during cooking (i.e. heat treatment) of red and yellow vegetables was 13-*cis*-β-

Table 1. Provitamin A concentrations (μg per 100 g) in tomato products (Brand A)

Product/sample			Before isomer separation	After isomer separation		
			β-carotene fraction	13-mono-*cis*-β-carotene	all-*trans*-β-carotene	9-mono-*cis*-β-carotene
Juice	Bottled	1	250	2	230	nd[a]
		2	260	2	230	nd
		3	160	1	149	nd
	Cartoned	1	406	13	308	nd
		2	444	106	207	28
		3	663	207	398	46
Purée	Bottled	1	683	123	435	nd
		2	846	133	569	nd
		3	426	48	295	nd
	Canned	1	407	114	242	23
		2	537	141	340	23
		3	1293	380	723	94
Paste	Bottled	1	927	293	510	55
		2	1337	154	1129	18
		3	1169	122	980	nd
	Canned	1	1038	193	519	nd
		2	990	28	751	nd
		3	1379	380	723	nd
Ketchup	Bottled	1	410	33	283	56
		2	544	15	432	83
		3	485	46	340	65

[a] nd, not detected.

Table 2. Provitamin A concentrations (μg per 100 g) in tomato products (Brand B)

Product/sample			Before isomer separation	After isomer separation		
			β-carotene fraction	13-mono-*cis*-β-carotene	all-*trans*-β-carotene	9-mono-*cis*-β-carotene
Purée	Cartoned	1	828	248	484	61
		2	826	113	610	62
		3	687	183	400	55
	Bottled	1	549	36	465	21
		2	893	110	679	56
		3	1057	247	725	52
	Canned	1	522	116	342	47
		2	494	132	311	34
		3	706	120	507	58
Paste	Bottled	1	877	144	544	75
		2	1016	132	707	44
		3	791	88	525	42
	Canned	1	805	261	342	107
		2	906	288	443	92
		3	914	226	510	95
Ketchup	Bottled	1	472	52	340	49
		2	581	64	418	61
		3	426	47	306	65

Table 3. Vitamin A values (RE per 100 g) of tomato products (Brand A)

Product/sample			Vitamin A value				% Overestimation		
			Without isomer separation	With isomer separation					
				a	b	c	a	b	c
Juice	Bottled	1	43	38	40	40	10	5	5
		2	44	38	40	40	13	8	8
		3	27	25	26	26	8	4	4
	Cartoned	1	68	52	55	55	31	24	24
		2	74	46	46	52	61	57	42
		3	111	87	90	99	28	23	12
Purée	Bottled	1	114	83	86	91	37	33	25
		2	141	107	110	115	32	28	23
		3	71	53	55	57	34	29	25
	Canned	1	68	52	53	58	31	28	17
		2	90	71	73	79	27	23	14
		3	216	160	165	182	35	31	19
Paste	Bottled	1	155	114	118	130	36	31	19
		2	223	203	211	217	10	6	3
		3	195	174	181	185	12	8	5
	Canned	1	173	104	107	114	66	62	52
		2	167	128	133	134	29	24	23
		3	230	154	159	172	49	45	34
Ketchup	Bottled	1	68	54	56	59	26	21	15
		2	91	79	82	85	15	11	7
		3	81	65	67	71	25	21	14

a, calculated according to the biological activities obtained by Deuel *et al.* (1944, 1945).
b, calculated according to the biological activities obtained by Deuel *et al.* (1944, 1945), corrected for loss on column.
c, calculated according to the biological activities obtained by Sweeney and Marsh (1973), corrected for loss on column.

carotene, with the exception of some samples of ketchup. In green vegetables, the major *cis*-isomer produced was shown to be 9-*cis*-β-carotene.

As would be expected the mild processing of tomato juice produced only 1–2 μg g^{-1} of 13-*cis*-β-carotene and no detectable 9-*cis*-β-carotene. Varying and appreciable levels of the *cis*-isomers were found in tomato purée and paste, regardless of the packaging material (carton, glass or can). Brand A had generally lower levels but a wider range with 13-*cis*-β-carotene ranging from 13 to 380 μg g^{-1} and the 9-*cis*-isomer from not detected to 94 μg g^{-1}. In Brand B, the corresponding ranges were 36–288 and 21–107 μg g^{-1}. This indicated that the processing of Brand A was generally milder, but the processing of Brand B was better controlled, resulting in more uniform products. Ketchup of both brands showed generally lower and similar concentrations of the *cis*-isomers.

When the vitamin A values were calculated, an overestimation of 8–13% was observed in tomato juice when the isomers were not quantified separately (Table 3). In tomato paste and purée, the overestimation for Brand A varied from 10 to 66% and for Brand B from 12 to 54% (Tables 3 and 4). For ketchup, the range for both brands was 15–26%.

Recovery of *trans*-β-carotene from the MgO/Hyflo-supercel column was reported by Sweeney and Marsh (1970) to be 98%, this was repeatedly confirmed in our laboratory at β-carotene levels which approximate those encountered in food samples. In three trials, recovery of β-carotene from the $Ca(OH)_2$ column utilised to separate the isomers was found to be 96%, thus in agreement with the 96% recovery observed by Sweeney and Marsh with the $Mg(OH)_2/Ca(OH)_2$ column used for the same purpose. Isomerisation on the column was not observed in our work and in that of Sweeney and Marsh.

If the 4% loss on the $Ca(OH)_2$ column were taken into consideration, the overestimations incurred when isomer separation was not accomplished would be lowered to 4–8% for tomato juice; 6–62% for tomato purée and paste, Brand A; 8–51% for tomato puree and paste, Brand B; 11–21% for ketchup of both brands (Tables 3 and 4).

In the two sets of calculations discussed above, the currently accepted biopotencies (53% for 13-*cis*-β-carotene, 38% for 9-*cis*-β-carotene, 100% for *trans*-β-carotene) were utilised. These activities were based on the rat assays carefully carried out by Deuel *et al.* (1944, 1945), monitoring gain in weight. Weight gain, however, is not a very sensitive and specific indicator. In a more recent paper, Sweeney and Marsh (1973) suggested biopotencies of 74% for 13-*cis*-β-carotene and 61% for 9-*cis*-β-carotene, based on vitamin A liver

Table 4. Vitamin A values (RE per 100 g) of tomato products (Brand B)

Product/sample			Vitamin A value				% Overestimation		
			Without isomer separation	With isomer separation					
				a	b	c	a	b	c
Purée	Cartoned	1	138	106	110	121	30	25	14
		2	138	116	120	126	19	15	9
		3	115	86	89	98	34	29	17
	Bottled	1	92	82	85	87	12	8	6
		2	149	126	131	137	18	14	9
		3	176	146	151	162	20	17	9
	Canned	1	87	70	73	78	24	19	11
		2	82	66	68	74	24	21	11
		3	118	99	102	109	19	16	8
Paste	Bottled	1	146	108	112	120	35	30	22
		2	169	132	137	143	28	23	18
		3	132	98	102	106	35	29	24
	Canned	1	134	87	89	102	54	51	31
		2	151	105	108	122	44	40	24
		3	152	111	114	126	37	33	21
Ketchup	Bottled	1	79	64	67	70	23	18	13
		2	97	79	85	90	23	14	8
		3	71	59	61	65	20	16	9

a, calculated according to the biological activities obtained by Deuel *et al.* (1944, 1945).
b, calculated according to the biological activities obtained by Deuel *et al.* (1944, 1945), correlated for loss on column.
c, calculated according to the biological activities obtained by Sweeney and Marsh (1973), corrected for loss on column.

storage in rats. If these latter biopotencies were used instead in the calculation of the vitamin A values, corrected for column losses, the overestimations due to non-separation of isomers would be lowered further to 3-52% for tomato purée and paste, brand A; 6–31% for tomato purée and paste, Brand B; 7–15% for ketchup of both brands.

The results obtained demonstrate that the *cis*-isomers of provitamin are present in foods at markedly variable levels, reaching appreciable amounts in some samples. Thus, notwithstanding the difficulties in its execution, isomer separation is necessary in some foods. However, the extent of overestimation when this separation is not carried out depends on how the calculation is accomplished. Before this difficult and error-prone step is made obligatory in official methods, several research needs will have to be dealt with. New assays, using more modern techniques (e.g. radioactive tracers) should be undertaken to obtain better values for the biopotencies in man. On the analytical aspect, more efficient HPLC columns are needed to separate the *cis*-isomers of β-carotene and other provitamins (e.g. α-carotene, β-cryptoxanthin) reproducibly. Whether an HPLC or an open column method is used, the absorption coefficients ($A^{1\%}_{1\,cm}$) of the provitamin A isomers in common solvents should be reconfirmed, since some discrepancies exist in the literature, or determined for those for which this value is not available (e.g. *cis*-β-cryptoxanthin). Because of the variability of the *cis*-isomer levels, especially of processed foods, when these data are finally required for food composition tables, a large number of samples for each food will have to be analysed to obtain more representative values.

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