papers and notes on methodology

Separation and quantitation of serum β-carotene and other carotenoids by high performance liquid chromatography

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Abstract We describe a specific assay for serum provitamin A (α- and β-carotene) by high pressure liquid chromatography (HPLC). The system separates α- and β-carotene in 7.4 min using a C_{18} μBondapak, 10-μm particle size column with a mobile phase of acetonitrile–chloroform 92:8 at 2 ml per min and a 462 nm detector. The HPLC assay had a recovery of 94.8% of added β-carotene and, at a serum concentration of 215.2 μg/L, had within-run and between-run precisions of 3.1% and 3.6%, respectively. In 65 subjects, the HPLC-determined provitamin A (α- and β-carotene) value was 343 ± 166 μg/L and averaged 23.4 ± 7.9% (range 9–43%) of the values obtained by a traditional colorimetric assay for total serum "carotenes." Although total serum carotenes showed no relationship to serum vitamin A ($r = -0.048$; $P = 0.78$), HPLC-determined α- and β-carotene was significantly inversely correlated ($r = -0.357$; $P = <0.05$).—**Katrangi, N., L. A. Kaplan, and E. A. Stein.** Separation and quantitation of serum β-carotene and other carotenoids by high performance liquid chromatography. *J. Lipid Res.* 1984. **25:** 400–406.

Supplementary key words fractionated carotenoids • β-carotene

Carotenoids are members of the terpenoid family of compounds which are characterized by their polyunsaturated nature. The most common terpenoids in nature are carotenes (α, β, γ, and δ), lycopene and some alcohol derivatives, such as xanthophyll, zeaxanthin, lycophyll, and cryptoxanthin. Carotenoids are synthesized by all plants and by some animals but not by humans, whose source of carotenes is dietary, from fruits and legumes. The most quantitatively important blood carotenoids are β-carotene, lycopene, and xanthophyll.

Not all carotenoids are provitamin A; that is, not all can be enzymatically converted to retinol or vitamin A. Only β-carotene is considered 100% provitamin A because each molecule of β-carotene yields, by oxidative cleavage, two molecules of vitamin A (retinol). The α and γ carotenes and cryptoxanthin are 50% provitamin A, while all other carotenoids are totally inactive as precursors of vitamin A.

Humans, as well as animals, are inefficient utilizers of carotenes. As β-carotene has a retinol equivalent value only 1/6 that of *trans*-retinol, about six times more β-carotene than preformed vitamin A is required in the diet to maintain normal dark adaptation in adults. Most clinical interest in the retinoids and carotenoids has, in the past, been related to the rare gross excess or deficiency states. More recently, blood retinol and dietary β-carotene have been inversely correlated with the risk of cancer (1–7). While serum retinol and retinol binding protein assays are specific for these substances, the current measurement of serum carotenes is totally nonspecific, quantitating all organic solvent-extractable material (known as carotenoids) which absorbs at a given wavelength, usually 450 nm. However, it is still unclear as to whether β-carotene itself or some other carotenoid(s) ingested simultaneously can effect cancer risk. It is known that β-carotene, although the most effective, is only one of a number of singlet oxygen quenchers (8) that can reduce the risk of environmentally produced cancer in mice (9).

As there are numerous carotenoids with potential anticarcinogenic activity in serum, it appears increasingly es-

Abbreviation: HPLC, high performance liquid chromatography.

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sential to separate, identify, and quantitate individual carotenoids. This would in turn allow more reliable assessment of the role of β-carotene and other carotenoids in cancer prevention.

Isolation and identification of individual carotenoids in food substances has been performed by gas–liquid, thin-layer, and high performance liquid chromatography (HPLC) (10–12). Only very recently has HPLC been applied to plasma for isolation and quantitation of specific carotenoids by us (13) and others (14, 15).

In the current report, we describe an HPLC technique for the rapid separation and quantitation of α- and β-carotene (provitamin A) from other carotenoids in serum. Using this specific assay, we have derived reference values for serum α- and β-carotene in healthy adults and explored the relationship between α- and β-carotene, total carotenoids, and vitamin A.

METHODS

Total serum carotenoids

Carotenoids were first dissociated from serum proteins by the addition of 95% ethanol. They were then extracted into petroleum ether and the absorbance was read at 450 nm (16). Quantitation was made against a standard curve consisting of pure β-carotene (Sigma Chemical Co., St. Louis, MO).

Fractionated serum carotenoids

HPLC apparatus. A Waters HPLC system (Waters Associates, Inc., Milford, MA) was used for all studies. The system consisted of a 6000A solvent delivery system, a U6K universal liquid chromatography injector, a stainless steel 3.9 mm × 40 cm μBondapak C_{18} column of 10-μm particle size, a 450 model variable wavelength detector equipped with a tungsten lamp source and a strip chart recorder (Omniscribe B-5000).

Solvents. All solvents used were HPLC grade and all mobile phases were filtered through 0.5-μm pore size filters (Type FH-Millipore, Inc.).

Standards. β-Carotene, α-carotene, and lycopene were purchased from Sigma Chemical Co. The standards were tested for purity using the HPLC separation system, and a single peak for each standard was obtained.

Standard solutions[2]

β-Carotene

Both synthetic β-carotene and natural β-carotene were initially evaluated, and no chromatographic or spectral differences were observed between them.

Stock solution I. Two hundred and fifty mg of β-carotene was placed in a 100-mL volumetric flask. Chloroform (ARA grade) was added to dissolve the carotene and to bring the volume to 100 mL (conc = 2.5 g/L). This stock solution was then aliquoted into vials, sealed, and stored at −20°C.

Stock solution II. Stock solution I was further diluted (1:10) with chloroform to a final concentration of 250 mg/L. This solution was shown to be stable for 7 days when stored at 4°C.

Working solution A. A fresh working solution was prepared each day by diluting 1 mL of stock solution II to 100 mL with isopropanol for a β-carotene concentration of 2.5 mg/L.

α-Carotene

Stock solution III. Five mg of α-carotene was placed in a 50-mL volumetric flask. Chloroform was added to dissolve the carotene and to bring the volume to 50 mL (conc = 100 mg/L), aliquoted into vials, sealed, and stored at −20°C.

Working solution B. Stock solution III was diluted with isopropanol to a final concentration of 2.5 mg/L. A fresh working solution was made daily.

α- And β-carotene standard (90% β isomer and 10% α isomer)

β-Carotene (stock solution I) (0.9 mL) and 2.5 mL of α-carotene (stock solution III) were placed in a 10-mL volumetric flask and brought to volume with chloroform. This solution was further diluted with isopropanol (total volume 100 mL) to give a concentration of β- and α-carotene of 25 mg/L (stock solution IV) and stored at 4°C for 7 days.

Working solution C. Stock solution IV was diluted tenfold with isopropanol (conc = 2.5 mg/L) on a daily basis.

Lycopene

Working solution D. One mg of lycopene was weighed and dissolved in 10 mL of chloroform in a 10-mL volumetric flask (conc = 100 mg/L) and kept at 4°C. A fresh working solution was made daily by diluting the lycopene stock solution 20-fold in isopropanol to give a concentration of 5 mg/L.

Analysis of serum samples

All blood samples were collected in aluminum foil-wrapped tubes and further preparations were carried out in subdued light. Analysis was by the method of standard addition. Each sample was split into two 1.0-mL aliquots. To one aliquot, 20 μL of stock solution IV (the provitamin A standard) was added. One mL of ethanol was then added dropwise to both tubes while vortexing, followed by 2 mL of petroleum ether. The tubes were capped, vortexed for 4–5 min, and centrifuged at 1500 rpm for 5 min at ambient temperature. Then 1.5 mL of the organic solvent (top) layer was carefully pipetted off and

[2] As carotenes are light and air sensitive, the preparation of standard solutions was carried out in the dark and the prepared solutions were stored at 4°C under N_2 in brown, aluminum foil-wrapped bottles.

placed into 10 mm × 100 mm tubes also wrapped in aluminum foil.

For colorimetric assessment of total carotenes, the absorbance of the petroleum ether layer was measured at 450 nm and the carotene concentration was calculated by comparison to a standard. For the HPLC analysis, 1.5 mL of the petroleum ether extract was evaporated to dryness under N_2 at ambient temperature and the residue was reconstituted in 150 μL of isopropanol.

Fifty μL of the reconstituted sample or the provitamin A working standard was injected into the HPLC system. The mobile phase was acetonitrile–chloroform 92:8 with a flow rate of 2 mL/min. Detection was at 462 nm (AUFS = 0.01) and chart speed 0.5 cm/min.

Calculation. P.H. = peak height from baseline.

$$\text{Recovery \%} = \frac{\text{P.H. spiked} - \text{P.H. unspiked}}{\text{P.H. standard}} \times \text{conc. standard} \times \frac{1}{10} \times \frac{2}{1} \times \frac{100}{0.5}$$

$$= \frac{\text{P.H. spiked} - \text{P.H. unspiked}}{\text{P.H. standard}} \times \text{conc. standard} \times 40.$$

$$\text{Serum concentration (mg/L)} = \frac{\text{P.H. unspiked}}{\text{P.H. standard}} \times \text{conc. standard} \times \frac{1}{10} \times \frac{2}{1} \times \frac{100}{\text{recovery}}$$

$$= \frac{\text{P.H. unspiked}}{\text{P.H. standard}} \times \frac{\text{conc. standard}}{\text{recovery}} \times 20 \text{ mg/L}.$$

Precision studies. Within- and between-day precision studies were carried out using a serum pool, aliquoted and frozen at −20°C.

Clinical studies. Serum from 65 healthy subjects was analyzed. The first group (n = 26) was comprised of laboratory volunteers aged 20–40 years in whom the values between the HPLC method and the colorimetric method were compared (Group A, **Table 1**). The second group consisted of 39 older (40–65 years), slightly overweight subjects with mild hyperlipidemia in whom the concentrations of serum provitamin A (α- and β-carotene) and total carotenoids were compared with serum vitamin A concentrations (Group B, Table 1).

Retinol (vitamin A) analysis. Serum vitamin A was analyzed by HPLC using a slight modification of the method described by Catignani and Bieri (17). The modifications included the use of a Waters 10-μm Radial Pak C_8 reversed-phase column in a Waters RCM. The mobile phase consisted of methanol–water 92:8 with a flow rate of 2

TABLE 1. Serum concentrations of total carotenoids, provitamin A by HPLC, vitamin A and plasma lipid levels in 65 subjects (mean ± SD)

Group	Age	N	Sex	Provitamin A	Total Carotenoids	Vitamin A	Cholesterol	Triglyceride	HDLC[a]	LDLC[b]	QI[c]
	yr			*μg/L*	*μg/L*	*μg/L*	*mg/dL*	*mg/dL*	*mg/dL*	*mg/dL*	
A. Under 40	33 ± 5	8	M	331 ± 191	1634 ± 515						
(normal volunteers)	28 ± 8	18	F	351 ± 164	1500 ± 326						
B. Over 40	51 ± 7	25	M	303 ± 153	1594 ± 622	531 ± 90	235 ± 46	170 ± 46	46 ± 7	163 ± 39	2.6 ± 0.2
(mildly obese and hyperlipidemic)	57 ± 6	14	F	416 ± 170	1700 ± 523	456 ± 131	310 ± 90	246 ± 167	46 ± 13	207 ± 57	2.8 ± 0.4

[a] High density lipoprotein cholesterol.
[b] Low density lipoprotein cholesterol.
[c] Quetelet Index = $\frac{\text{weight (grams)}}{[\text{height (cm)}]^2}$.

mL/min. The standard curve was prepared by adding aliquots of ethanolic retinol and retinol acetate (internal standard) to plasmanate (Cutter Laboratory, Berkeley, CA).

RESULTS

Both normal and reverse stationary phases were initially assessed. The normal phase (μPorasil) with hexane did not produce adequate separations; the reverse phase (μBondapak C_{18}, 10-μm particle size) resulted in separation of α- and β-carotenes from other carotenoids. After selecting the μBondapak C_{18} column, two different mobile phases were evaluated: methanol–isopropanol 80:20 and acetonitrile–chloroform 92:8. The alcoholic mobile phase resulted in separation of the alcohol derivatives which eluted faster than carotenes but did not separate carotenes from lycopene, a major carotenoid in serum. The acetonitrile–chloroform mobile phase resulted in superior separation of the carotenes from other carotenoids.

Standard curve

Fig. 1 shows the chromatogram of standards: lycopene and β- and α-carotene 90:10. The retention times were 5.2 min for lycopene and 7.4 min for β-carotene. α-Carotene appears as a small shoulder peak at 7.2 min.

A standard curve using the provitamin A standard (solution IV) showed the assay was linear to 2500 μg/L.

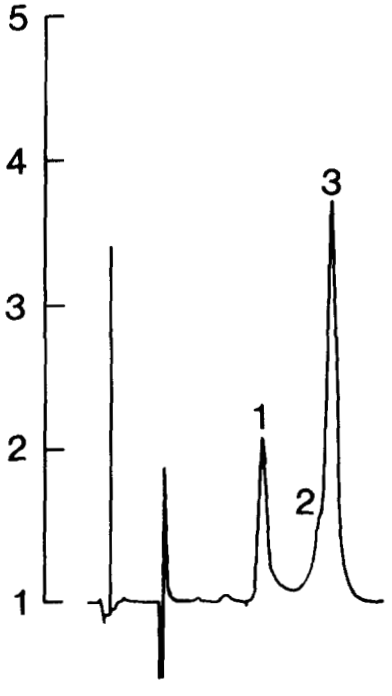

Fig. 1. HPLC elution of standards: 1, lycopene at 5.2 min; 2, α-carotene at 7.2 min; and 3, β-carotene at 7.4 min. Vertical axis shows relative peak height. Horizontal axis indicates retention time in minutes.

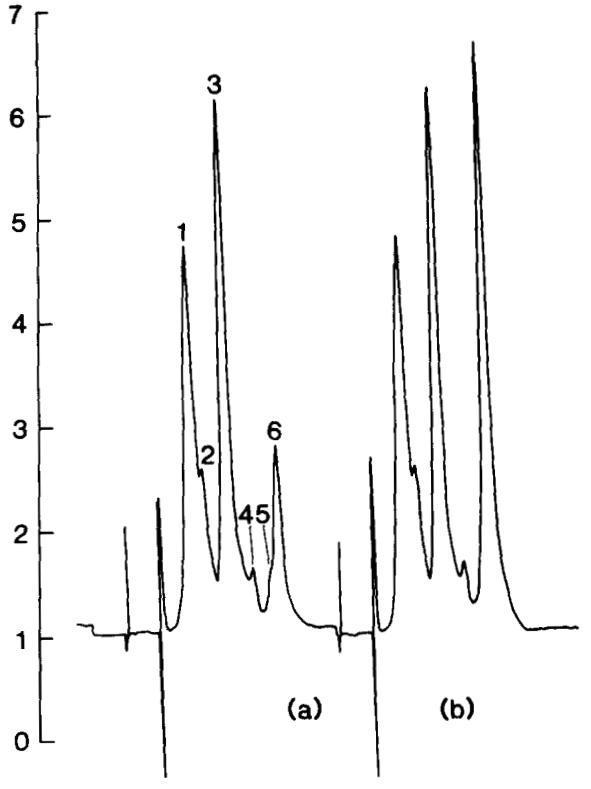

Fig. 2. HPLC elution profile of serum carotenoids without (a) and with (b) addition of α/β carotenoid (10:90) standard. Vertical axis indicates relative peak height. Horizontal axis indicates relative retention time. Peaks 1 and 2, unidentified polar carotenoids; peak 3, lyocopene; peak 4, δ- and γ-carotenes; peak 5, α-carotene; peak 6, β-carotene.

Serum carotenoids

Fig. 2a and b shows chromatograms of unspiked and spiked serum samples, respectively. The early (<4.3 min) eluting peaks are probably alcohol derivatives, such as xanthophyll and zeaxanthin. Lycopene appears as peak 3, α-carotene as peak 5, while the sixth peak is β-carotene. In order to confirm that the HPLC method did, in fact, contain the major provitamin A, β-carotene, a fraction was collected from the downslope of peak 6 (Fig. 2a). This isolated fraction was then identified by two additional procedures. Using a Perkin-Elmer dual-beam scanning spectrophotometer, the sample was scanned from 400 through 500 nm. **Fig. 3** shows the spectral scan of a pure β-carotene standard (stock solution II) and the isolated HPLC–β-carotene fraction, respectively. They appear identical with two maxima of absorption at 493 and 462 nm. The ratios of absorbances at 493 nm to 462 nm were similar (β-carotene standard, 0.765; HPLC peak, 0.766). Spectral scans of the other peaks showed different maxima and ratios.

The sample obtained from HPLC fraction 6 was also analyzed by mass spectrometry (18) employing direct injection. Comparison was made with the β-carotene (stock

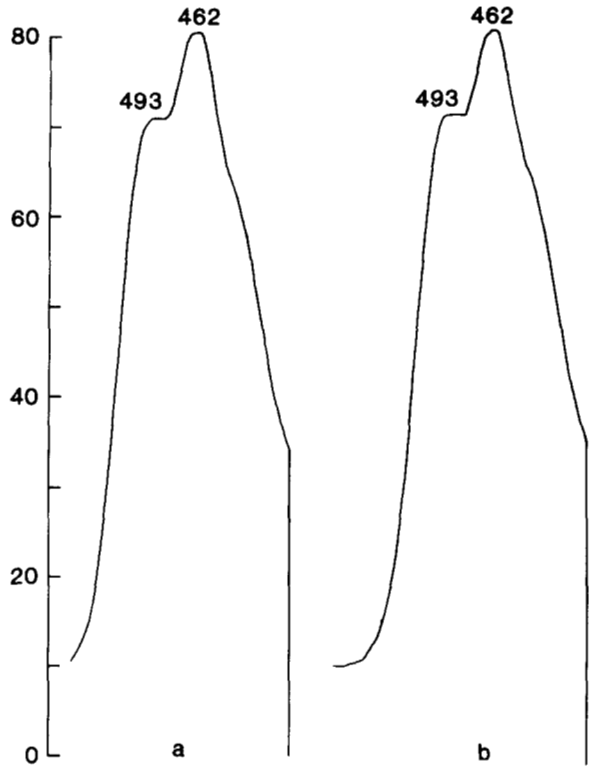

Fig. 3. Absorption spectra of the β-carotene HPLC fraction 6 (a) and the pure β-carotene standard (b). Vertical axis indicates relative peak heights.

solution II) and lycopene (working solution D) standards. Both the HPLC peak 6 and β-carotene samples showed the presence of mass ions at 444 and an absence of the ions at 430 which characterized the lycopene standard.

Recovery and reproducibility

To assess recovery, values were determined after addition of a known concentration of the provitamin A standard to the serum of the 26 normal volunteers. The recovery of provitamin A by the HPLC assay was 94.8 ± 4.1% (mean ± SD) with provitamin A concentrations ranging from 114 to 738 μg/L. The recovery by the colorimetric assay (total carotenoids) was 99.0 ± 3.1% (mean ± SD) over a total carotenoid range of 1090 to 2454 μg/L. Within-run precision (n = 12) at a serum α- and β-carotene concentration of 215.2 μg/L was 3.1%, while between-day precision (n = 12) at the same level was 3.6%.

Clinical studies

Comparison with the colorimetric method. Table 1 shows the results obtained on 65 serum samples. The HPLC provitamin A assay as a percent of the colorimetric assay was 23.4 ± 7.9%, while the area of the provitamin A peak on HPLC (peaks 5 and 6) was 20.5 ± 7.8% of the total area of all peaks of the chromatogram (peaks 1–6, Fig. 2). Regression analysis of the HPLC provitamin A area/total chromatogram and HPLC provitamin A value/colorimetric value showed $r = 0.792$ ($P < 0.001$), slope 0.80, and intercept 6.9%. The correlation between provitamin A by HPLC and the colorimetric procedure in all 65 subjects was $r = 0.699$ ($P < 0.001$), with a slope and intercept of 0.23 and −21 μg/L, respectively.

Comparison of HPLC provitamin A and HPLC vitamin A levels. Serum provitamin A and vitamin A levels were determined by HPLC in 39 older, slightly obese subjects (Group B, Table 1). Regression analysis (**Fig. 4**) between serum provitamin A and vitamin A showed a significant ($P < 0.05$, $r = -0.357$) negative correlation. Similar analysis between total carotenoids and vitamin A (**Fig. 5**) failed to demonstrate any significant relationship ($r = -0.048$, $P = 0.78$). In 32 of 39 subjects (81%), the serum vitamin A concentration exceeded the provitamin A level. The mean serum provitamin A concentration was 57% and 91% of the mean serum vitamin A level (Table 1) in males and females, respectively.

In order to determine if the provitamin A levels were age- and sex-dependent, a *t*-test of means which assumed equal variances was performed comparing each of the four groups with each other (Table 1). Only the comparison between men and women over 40 years old tended toward significance ($P = 0.058$).

DISCUSSION

After years of analytical inactivity in serum carotenoids, there is renewed interest in specific assays for carotenoids, especially β-carotene, as reflected by the recent publications by three different groups (13–15). Although the assays by Broich, Gerber, and Endman (14) and Driskell, Bashor, and Neese (15) are similar to those described in this paper, there are significant differences (**Table 2**).

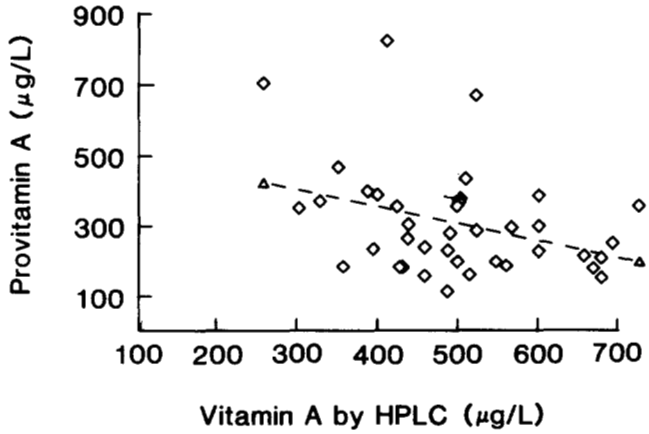

Fig. 4. Comparison of serum provitamin A and vitamin A by HPLC in 39 subjects.

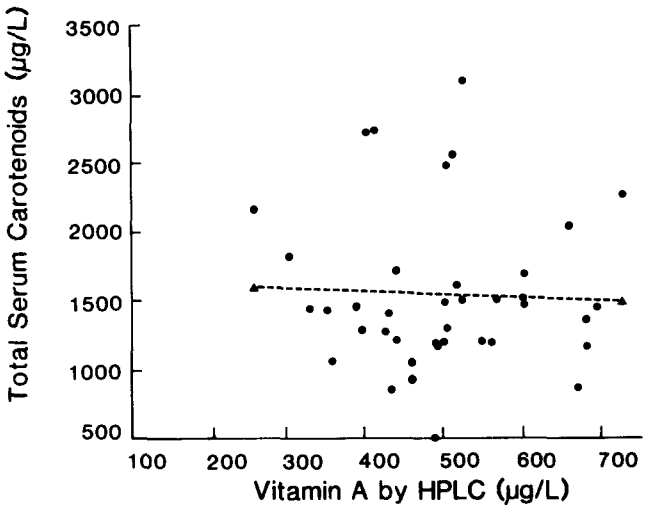

Fig. 5. Comparison of serum total carotenoids and vitamin A in 39 subjects.

The present assay is more rapid than the other two procedures. For example, provitamin A (α- and β-carotene) is resolved in less than half and two-thirds the time taken for the assays of Broich et al. (14) and Driskell et al. (15), respectively. This may be an important feature in large epidemiological studies where sample throughput per hour can reduce costs significantly. The reproducibility of our assay, both within- and between-batch, appears significantly better than the other two procedures, especially considering that the provitamin A concentration of the sample used in assessment was one-third to one-quarter that used in the other studies. In studies where low levels of α- and β-carotene may have clinical or epidemiological significance, it is obviously important to demonstrate good precision as well as accuracy. This has been achieved in the present study.

Numerous previous procedures for serum carotenoids have demonstrated that it is extremely difficult to ensure that only α- and/or β-carotene is being measured. Even for HPLC procedures, a single peak may contain more than one analyte as seen in Figs. 1 and 3 where α-carotene appears as a shoulder of the β-carotene peak. To ensure the contents of each peak, it is therefore necessary to confirm it, not only by obtaining an increase in peak height by addition of a standard, but by at least one other more definitive technique. The present study does this by using both differential visible spectrometry and mass spectrometry.

There is a significant correlation between serum total carotenoids and provitamin A (α- and β-carotene); however, in any given individual the use of total carotenoids as an indicator of α- and β-carotene concentration can be grossly misleading. The ratio of α- and β-carotene to total carotenoids in the subjects studied varied in excess of fourfold (9.6% to 43.6%). In addition, it is possible that clinical studies in which serum α- and/or β-carotene levels are doubled may result in small and perhaps non-significant increases in total carotenoid concentrations. This may obscure the study interpretation and potential conclusions.

In the current study, only 9–43% of total serum carotenoids were α- and β-carotene. If there is an inverse association between dietary carotenoids and cancer, with the availability of improved methodology as in this report, we speculate that it would be important to longitudinally determine the relationships between serum β-carotene and cancer event rate. The relationship between cancer and serum α- and γ-carotenes and cryptoxanthin (all of which are 50% provitamin A), as well as the remaining common terpenoids, including lycopene and some of the alcohol derivatives, such as xanthophyll, etc., should also be determined. A second new finding in the current study was that serum α- and β-carotene levels are, on an average, substantially lower, not higher, than those of vitamin A. Within this frame of reference, the significant inverse correlation between β-carotene and vitamin A requires further confirmation and evaluation. Since both serum vitamin A levels and dietary carotene intake are inversely related to cancer, the finding of an inverse association between serum α- and β-carotene and vitamin A raises the question, speculatively, that other carotenoids, besides β-carotene, could be playing an important role relative to cancer.

TABLE 2. Comparison of three recent assays for serum β-carotene using HPLC

	Broich et al. (14)	Driskell et al. (15)	Present Method
Stationary phase	LC-18 (5 μm)	μBondapak C_{18} (10 μm)	μBondapak C_{18} (10 μm)
Mobile phase	CH_3CN/MeOH/$CHCl_3$ (47:47:6)	CH_3CN/CH_2Cl_2 (89:11)	CH_3CN/$CHCl_3$ (92:8)
Detection	466 nm	450 nm	462 nm
Retention time (min)			
Lycopene	9.26		5.2
α-Carotene	13.99		7.2
β-Carotene	15.01	11.6	7.4
Coefficient variation within run	6.4% at 860 μg/L	4.8% at 660 μg/L	3.1% at 215 μg/L
β-Carotene between run		5.7% at 660 μg/L	3.6% at 215 μg/L

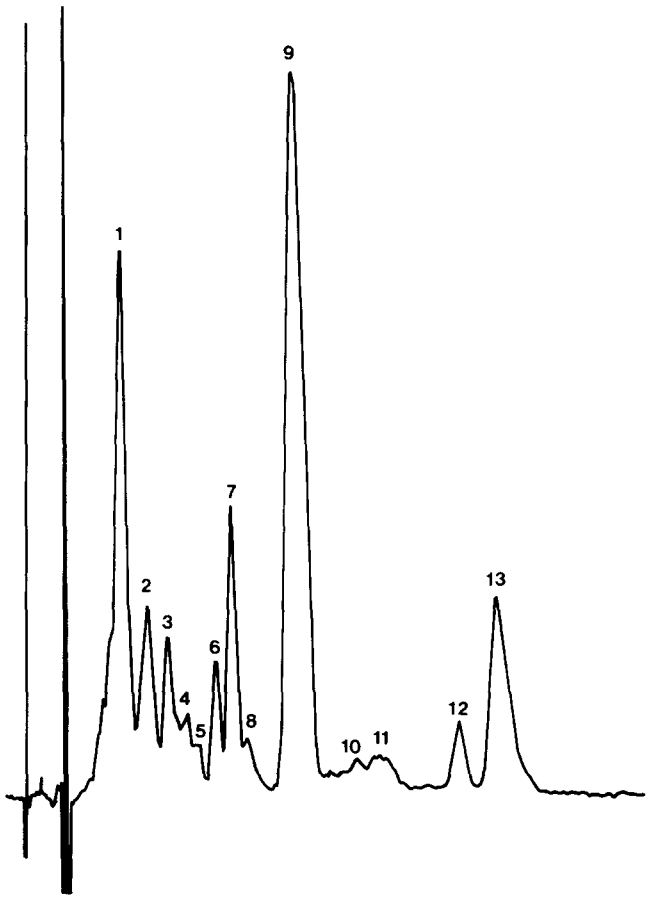

Fig. 6. Expanded HPLC assay for isolation of additional serum carotenoids. Peak 1, xanthophyll; peaks 2–8, unidentified carotenoids; peak 9, lycopene; peak 10, δ-carotene; peak 11, γ-carotene; peak 12, α-carotene; peak 13, β-carotene.

To this end, we have continued to expand the carotenoid profile eluting prior to α- and β-carotene and to separate α- and β-carotene more clearly. By altering the mobile phase to acetonitrile–water–isopropanol–chloroform 86:2.5:3.5:8 (v:v) and the column packing size to 5 μ, we have been able to detect at least eleven peaks in serum. While some of these peaks are known, a number still have to be identified. These changes have also produced baseline separation of α- and β-carotene (**Fig. 6**).

Further analytical advances in specific identification and quantitation of the individual carotenoids and cryptoxanthin, as well as the common terpenoids and the alcohol derivatives, should facilitate clinical and epidemiological studies of the relationship of the terpenoids and cancer.

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