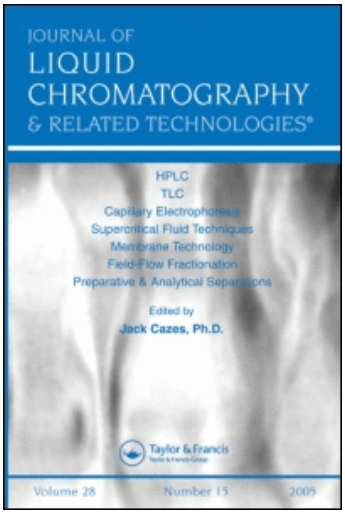

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A Rapid Separation of Ten Carotenoids, Three Retinoids, Alpha-Tocopherol and d-Alpha-Tocopherol Acetate by High Performance Liquid Chromatography and its Application to Serum and Vegetable Samples

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A RAPID SEPARATION OF TEN CAROTENOIDS, THREE RETINOIDS, ALPHA-TOCOPHEROL AND d-ALPHA-TOCOPHEROL ACETATE BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY AND ITS APPLICATION TO SERUM AND VEGETABLE SAMPLES

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ABSTRACT

The methods described herein were developed for the quantitative analysis of 10 carotenoids (lutein, zeaxanthin, canthaxanthin, beta-apo-8'carotenal, beta-cryptoxanthin, echinenone, lycopene, gamma-carotene, alpha-carotene, beta-carotene/15-cis-beta-carotene), 3 retinoids (retinol, retinyl acetate, retinyl palmitate) and alpha-tocopherol and d-alpha-tocopherol acetate with two-channel detection and a single sample preparation.

Two fast and simple methods are described using a Spheri-5-RP-18 column, with two different mobile phases—acetonitrile:dichloromethane:methanol (70:20:10) in 10 minutes and acetonitrile:methanol (85:15) in 25 minutes.

These methods would be beneficial to food scientists, cancer researchers and epidemiologists for the accurate estimation of vitamin A activity and the antioxidant capability of different compounds present in the human diet and the levels of these compounds in human serum.

INTRODUCTION

Epidemiologic evidence indicating the existence of a relationship between different compounds (nutrients and nonnutrients) present in the human diet and the incidence of certain diseases (e.g. various types of cancer, cardiovascular diseases, cataracts, etc.) (1-10) has led in recent years to increased interest in several components of the diet which remain to be fully characterized and quantified in food as well as in biological tissues (10-14).

These compounds include various carotenoids, tocopherol and ascorbic acid (WHO/MONICA Project). Several carotenoids can function as effective quenchers of singlet oxygen and can block free radical-mediated reactions, acting as protective agents per se, rather than by conversion into retinol (5,8,15).

Intake of carotenoid and ascorbic acid-rich food is associated with a lower risk of lung cancer and, depending on the study, has a significant effect on the risk of other cancers or of all cancers (4,5,7, 16). In this respect, the carotenoid most studied from the epidemiologic point of view is beta-carotene, and protective associations have been noted between this substance and lung cancer. Nevertheless, other carotenoids could prove to be of equal or greater value in the prevention of cancer; early experiments have

demonstrated weaker protective associations between lycopene and cancers of pancreas and bladder; canthaxanthin was also effective in preventing skin cancers and several chemical-induced cancers (7,9,15,17). However, not all the results support an anticarcinogenic function for carotenoids, and it is appropriate to begin defining the limits of the hypothesis, as other authors (18,19) have recommended. The epidemiologic evidence can only be tested if clinical studies incorporate more discriminatory analyses of carotenoids in food and tissues.

With regard to food composition, the current tables report only total vitamin A activity or carotene content, calculated from data obtained by direct spectrophotometric or open-column chromatography techniques. In fact, many edible fruits and vegetables appear to be poor sources of beta-carotene but relatively good sources of other carotenoids (20,21).

On the other hand, most determinations of carotenoids in serum using high performance liquid chromatography (HPLC) are focused on the quantification of beta-carotene and, less frequently, measurement of alpha-carotene, lycopene, cryptoxanthin and lutein plus zeaxanthin can be found (22-28).

The methods we describe here permit the simultaneous determination of carotenoids with provitamin A activity in humans, such as beta-carotene, alpha-carotene, gamma-carotene, beta-cryptoxanthin and beta-apo-8'carotenal; and some nonprovitamin carotenoids, of interest because of their antioxidant activity (e.g. canthaxanthin); as well as lycopene, lutein and zeaxanthin, given their presence in frequently consumed vegetables; and echinenone as a possible inter-

nal standard. These HPLC systems with two-channel detection allow the simultaneous separation of retinol, retinyl acetate, retinyl palmitate, d-alpha-tocopherol and tocopherol acetate in serum and vegetable samples.

MATERIALS

Lycopene, all-trans-alpha and beta-carotene, all-trans-retinol, retinyl acetate, retinyl palmitate, d-alpha-tocopherol and d-alpha-tocopherol acetate standards were obtained from Sigma Chemical Co. (St. Louis, Mo.). Beta-apo-8'carotenal was obtained from Fluka Chemie AG (Switzerland). Gamma-carotene, canthaxanthin, beta-cryptoxanthin, 15-cis-beta-carotene, echinenone, lutein and zeaxanthin were gifts from Hoffman-La Roche (Basle, Switzerland). Stabilized tetrahydrofuran, hydroxybutyl-toluene, anhydrous granular sodium sulfate and dichloromethane were purchased from Carlo Erba (Spain). Methanol, ethanol, n-hexane and acetonitrile were supplied by Merck (Spain), and anhydrous magnesium carbonate powder by Scharlau (Spain).

METHODS

Preparation of Standards

Stock solutions of each carotenoid were prepared by dissolving 1-3 mg of the compound in 50 ml tetrahydrofuran. Standards were stored at -20°C and pro-

tected from light. These solutions were further diluted to provide working standards.

Stock solutions of retinoids, d-alpha-tocopherol and d-alpha-tocopherol acetate were prepared by dissolving different amounts in ethanol to obtain concentrations ranging from 0.05 to 1 mg/ml.

Concentrations were calculated on the basis of published absorptivity values (E 1% 1 cm) in hexane, ethanol or petroleum ether (29,30). Values used and the wavelength maxima were: lutein, 2550 at 445 nm; canthaxanthin, 2200 at 466 nm; beta-apo-8'carotenal, 2640 at 457 nm; beta-cryptoxanthin, 2386 at 452 nm; echinenone, 2158 at 458 nm; lycopene, 3450 at 472 nm; zeaxanthin, 2540 at 450 nm; alpha-carotene, 2800 at 444 nm; beta-carotene, 2592 at 453 nm; 15-cis-beta-carotene, 2340 at 450 nm (data from Hoffman-La Roche, Basle); gamma-carotene, 3100 at 462 nm; retinol, 1835 at 325 nm; retinyl acetate, 1565 at 328 nm; d-alpha-tocopherol, 72 at 292 nm; and tocopherol acetate, 40 at 285 nm. The concentrations of the stock solutions were recalibrated monthly.

Liquid Chromatographic System

High performance liquid chromatography was carried out in an ALC/GPC chromatograph (model 201, Waters Associates, Milford, Mass.) equipped with a model 6000 A pump, dual reciprocating piston heads, model U 6K septumless injector, and programmable multiwavelength detector, model 490 E. The detector was recorded on a M730 data module (Waters Associates).

Two different columns and two different solvent systems were used for the reversed-phase separations of carotenoids, retinoids, tocopherol and tocopherol acetate. These were as follows:

- System I: a 5 μm Spheri-5-RP-18 column (Brownlee Labs, Kontron Analytic), 220 x 4.6 mm, with a guard column of Aquapore ODS type RP-18, 15 x 3.2 mm, 7 μm. Solvent, acetonitrile:dichloromethane:methanol (70:20:10); flow rate, 1.8 ml/min. 5 - 10 μl were injected.
- System II: a 5 μm Spheri-5-RP-18 column (Brownlee Labs, Kontron Analytic), 220 x 4.6 mm, with a guard column of Aquapore ODS type RP-18, 15 x 3.2 mm, 7 μm. Solvent, acetonitrile:methanol (85:15); flow rate, 1.8 ml/min initially, to be increased to 3.5 ml/min, as shown in the figures. 5 - 10 μl were injected.

A similar column, 5 μm Spheri-5-ODS, 220 x 4.6 mm (Brownlee Labs, Kontron Analytic) was also assayed with the solvents described for systems I and II.

Carotenoids were detected at 450 nm, retinol and retinyl acetate at 313 nm, retinyl palmitate at 325 nm, and alpha-tocopherol and tocopherol acetate at 280 nm in both chromatographic systems.

Procedure

Serum samples (400 μl) were extracted three times with 2 ml of n-hexane. The organic layer was isolated and evaporated to dryness under vacuum and nitrogen, and the residue reconstituted with 300 μl of tetrahydrofuran. A 5 - 10 μl aliquot was injected into the column.

The procedure for extraction from plant origin is very similar to the one developed by Bushway and Wilson (31) with slight modifications.

RESULTS AND DISCUSSION

The aim was to define the best conditions for carrying out the separation and quantification of a mixture of carotenoids, retinoids, tocopherol and tocopherol acetate. For this purpose, we have employed two chromatographic columns and two mobile phases. The chromatographic profiles obtained with system I are represented in figures 1a, 1b and 2.

In figure 1a, the first peak corresponds to lutein and/or zeaxanthin, which do not resolve under the conditions set up in system I. The presence of 15,15'cis β-carotene can be detected at the downslope of the β-carotene peak, as seen in figure 1b, and is confirmed by the presence of a cis-peak at 340 nm. In figure 2 it can be observed that retinol, retinyl acetate, tocopherol, tocopherol acetate and retinyl palmitate resolve satisfactorily.

Under the chromatographic conditions described in system II, the separation of lutein and zeaxanthin is obtained, as is that of the remaining carotenoids except 15-cis-beta-carotene (figure 3). This elution system afforded a better separation of the polar carotenoids and also provided a good resolution of lutein and zeaxanthin under isocratic conditions. However, under isocratic elution, the separation of carotenoids with low polarity, such as β-carotene, is time-consuming and produces broad peaks. We improved

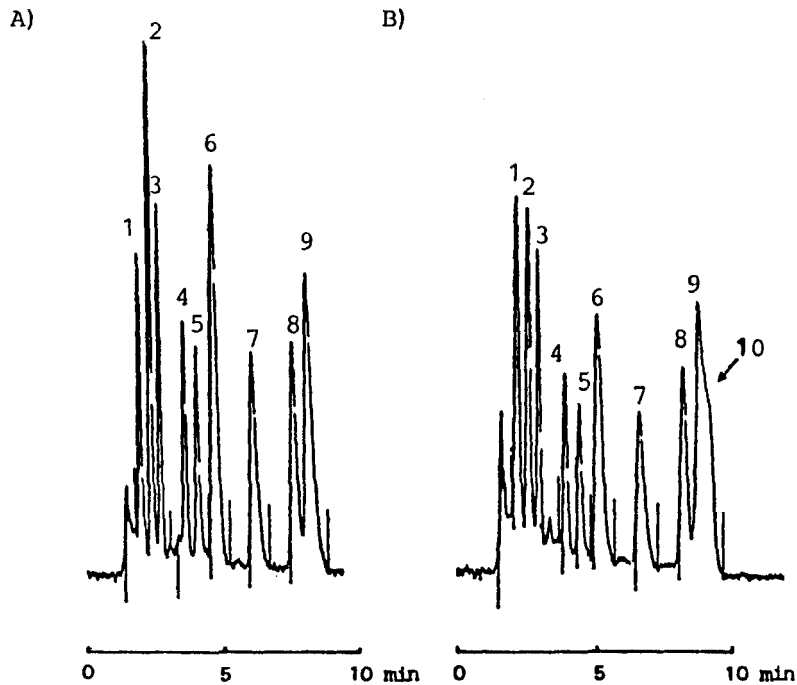

FIGURE 1.- Separation of carotenoid standards on Spheri-5 RP-18.
Mobile phase: acetonitrile/ Dichlorometane/ methanol (70:20:10). Flow rate: 1.8 ml/min. Detection at 450 nm (x 0.01 AUFS). Peak identification: 1, Lutein; 2, Cantaxanthin; 3, β-apo-8'carotenal; 4, β-cryptoxanthin 5, echinenona; 6, lycopene; 7, γ-carotene; 8, α-carotene; 9, β-carotene; 10, 15-cis-β-carotene.

the method by a programmed increase in flow rate, which shortened the retention time of the apolar carotenoids.

The chromatographic separation of lutein and zeaxanthin is not easily obtained with reverse phase C-18 columns (32), and it is usually necessary to employ elution gradients (33,34) or derivatization with fatty

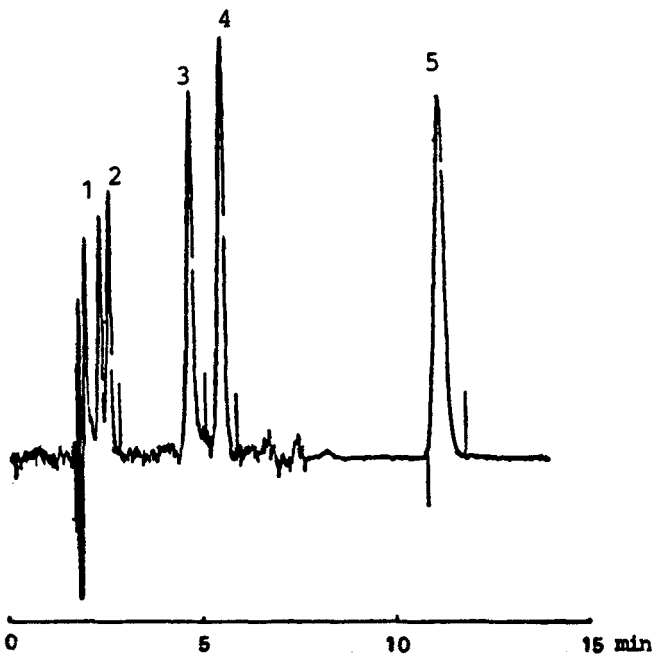

FIGURE 2.- Chromatogram of standard mixture. Chromatographic conditions: System I. Flow rate: 1.5 ml/min. Chart speed: 0.7 cm/min. Peak identification:
1, retinol (313 nm, x0.005 AUFS)
2, retinyl acetate (313 nm, x0.005 AUFS)
3, d-α-tocopherol (280 nm, x0.005 AUFS)
4, d-α-tocopherol acetate (280nm, x0.005 AUFS)
5, retinyl palmitate (325 nm, x0.03 AUFS)

acids and chromatography of their esters (32). Under isocratic conditions, Krinsky and Welankiwar (35) obtained the separation of lutein and zeaxanthin in a spinach sample, using a Lichrosorb RP-18 column and acetonitrile:methanol (85:15) as mobile phase, but they changed the mobile phase to obtain beta-carotene.

Under the isocratic conditions described for system II, the chromatographic separation of lutein and

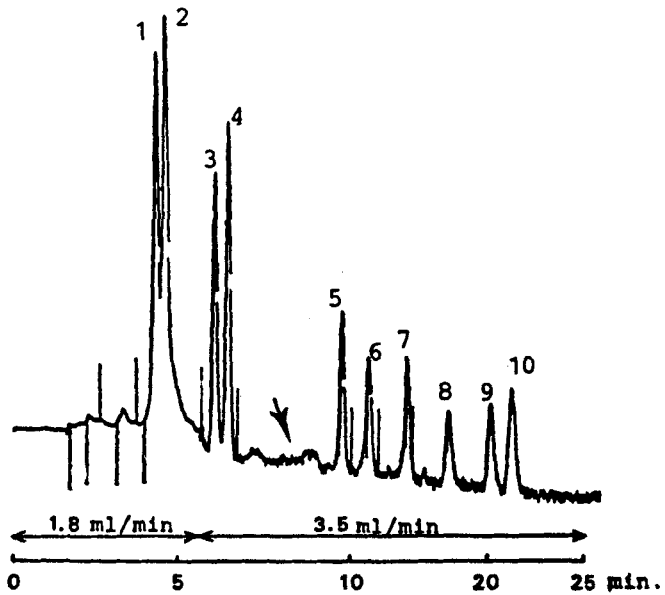

FIGURE 3.- Separation of carotenoids standards. System II. Detection 450 nm, x0.02 AUFS for 5min, and then x0.005 AUFS. Chart speed: 0.5 cm/min and 0.25 cm/min (arrow).
Peak identification: 1, lutein; 2, zeaxanthin; 3, canthaxanthin; 4, β-apo-8'carotenal; 5, β-cryptoxanthin 6, echinenona; 7, lycopene; 8 γ-carotene; 9, α-carotene 10, β-carotene.

zeaxanthin takes five minutes, and is largely determined by the volumes injected and the solvent into which they are injected. Thus, when 5 μl are injected into tetrahydrofuran, we obtain a good separation; however, 10 μl in the same medium is not effective, as can be seen in figure 4. This problem can be solved by injecting these compounds into ethanol, in which case the volume is not such a critical factor. The inconvenience of injecting the standards into ethanol

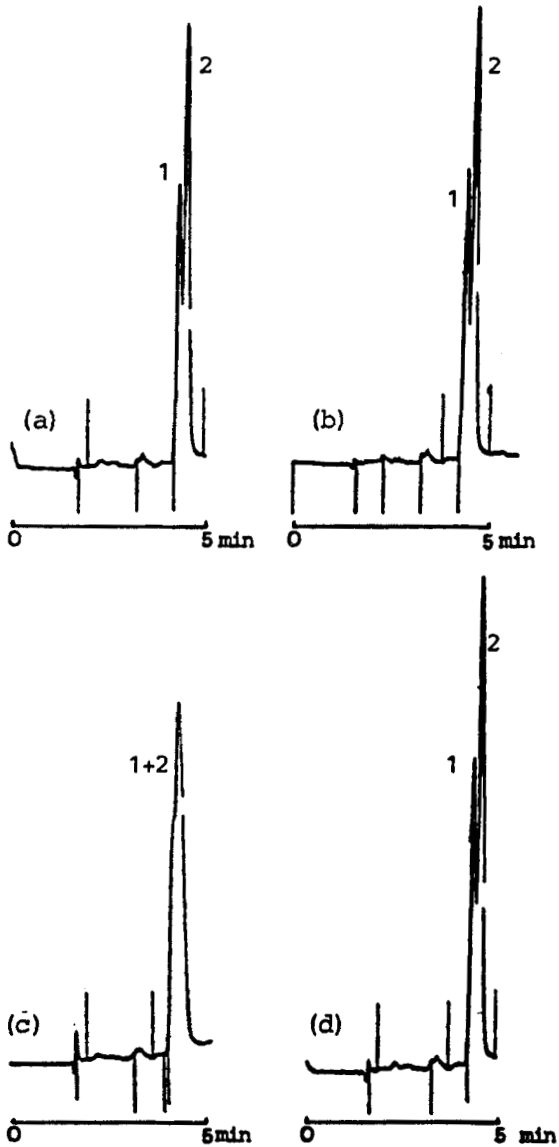

FIGURE 4.- Chromatographic profiles of a constant concentration of a mixture of lutein (1) and zeaxanthin (2) injected in ethanol: 5 µl (a), 10 µl (d); injected in tetrahydrofuran: 5 µl (b), 10 µl (c). System II.

is the excessive broadening of the peaks corresponding to the apolar carotenoids, making their quantification more difficult.

The Spheri-5-ODS (polyfunctional) column was assayed with the mobile phases described for both system I and II. With that of system I, the results obtained are not significantly different from those achieved with the Spheri-5-RP-18 (monofunctional) column. In contrast, with the mobile phase from system II , the chromatographic separation of lutein and zeaxanthin improves with respect to that achieved with the Spheri-5-RP-18 column in that the peaks resolve to baseline (figure 5), but the resolution of other standards declines.

The chromatographic constants for a mixture of standards in systems I and II are indicated in tables 1, 2 and 3. The correlation coefficients between the height of each peak and the concentration of the corresponding compound in the standard solution range between 0.996 and 0.999.

The detection limit of the different carotenoids has been determined in both systems by simultaneous injection of all the standards since in serum or vegetable samples they are all found together.

Under the chromatographic conditions in system I, at 450 nm, the standards injected into 10 μl tetrahydrofuran, approximately 0.3 ng of lutein, beta-apo-8'carotenal, canthaxanthin, β-cryptoxanthin, echinenone, gamma-carotene and alpha-carotene were detectable. Lycopene and beta-carotene were detectable from 1 ng on. The detection limit at 313 nm is 0.3 ng for retinol, 0.4 ng for retinyl acetate and 1.2 ng for retinyl palmitate (325 nm); at 280 nm, the d-alpha-

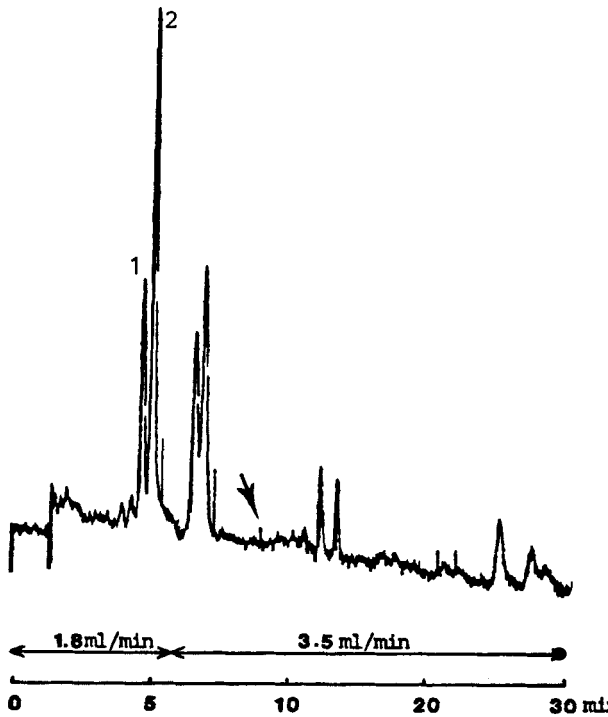

FIGURE 5.- Separation of carotenoid standards on Spheri-5 ODS column. System II. Detection 450 nm. Chart speed: 0.5 cm/min and 0.25 cm/min (arrow). Peak identification: 1, lutein; 2, zeaxanthin.

tocopherol and tocopherol acetate standards can be detected at amounts of approximately 15 ng.

System II affords less sensitivity for the detection of some carotenoids, the limit being 1 ng for beta-cryptoxanthin and echinenone (5 μl), 1 ng for zeaxanthin (2.5 μl), 2 ng for alpha-carotene (7.5 μl), and 4.5 ng for lycopene (5 μl) and beta-carotene (7.5 μl) when these standards are injected into tetrahydrofuran. When the standards are injected into ethanol

TABLE 1.-

Chromatographic Constants of Spheri-5-RP-18 (Kontron Analytic) High Performance Liquid Chromatographic Column, using Acetonitrile/ Dichloromethane/ Methanol (70/20/10) as Mobile Phase and a Flow Rate of 1.8 ml/min.

	Lutein	Canta-xanthin	β-apo-8´ carotenal	β-Cripto-xanthin	Echinenona	Lycopene	γ-carotene	α-carotene	β-carotene	K´	N	Range (ng)
					Separation factor (α)							
Lutein	——	1.333	2.333	5.333	7.333	8.000	10.000	14.500	14.222	0.428	711	0.44 - 318
Cantaxanthin	1.668	——	1.000	4.000	6.000	6.857	9.500	13.500	13.333	0.714	1024	0.39 - 642
β-apo-8´carot.	2.168	1.299	——	3.000	5.000	6.000	8.750	12.750	12.666	0.928	1296	0.30 - 585
β-Criptoxanthin	3.670	2.200	1.692	——	2.000	3.428	6.500	10.500	10.666	1.571	2304	0.42 - 346
Echinenona	4.672	2.801	2.155	1.273	——	1.714	5.000	9.000	9.333	2.000	3136	0.37 - 582
Lycopene	5.672	3.400	2.616	1.545	1.214	——	3.111	6.666	7.200	2.428	2304	1.31 - 420
γ-carotene	8.009	4.801	3.693	2.182	1.714	1.411	——	3.200	4.000	3.428	2460	0.53 - 540
α-carotene	10.679	6.401	4.925	2.913	2.285	1.882	1.333	——	1.090	4.571	3893	0.57 - 552
β-carotene	11.682	7.002	5.387	3.182	2.500	2.059	1.458	1.093	——	5.000	3136	1.07 - 415
					Resolution (R)							

Separation factor (α) = K'_2 / K'_1 ; W = peak width; Resolution (R) = $V_2-V_1 / \frac{1}{2}(W_2+W_1)$; V = retention volume; V_0 = void volume; Capacity factor (K')= $V-V_0/V_0$; Number of theoretical plates (N) = $16\ (V/W)^2$.

TABLE 2.-

Chromatographic Constants of Spheri-5-RP-18 (Kontron Analytic) High Performance Liquid Chromatographic Column, using Acetonitrile / Dichloromethane / Methanol (70/20/10) as Mobile Phase and a Flow Rate of 1.5 ml/min.

	Trans-retinol	Retinyl acetate	Tocopherol	Tocopherol acetate	Retinyl palmitate	K´	N	Range (ng)
				Separation factor (α)				
Trans-retinol	----	1.2121	9.428	12.285	20.000	0.333	1820	0.31 - 70
Retinyl acetate	1.500	----	7.631	10.263	18.275	0.500	1600	0.45 - 67
Tocopherol	5.124	3.416	----	2.500	12.830	1.708	4225	15.50 - 3262
Tocopherol acetate	6.375	4.250	1.244	----	11.166	2.125	5625	16.61 - 4986
Retinyl palmitate	14.748	9.832	2.878	2.313	-----	4.916	5041	1.26 - 396
				Resolution (R)				

Separation factor (α)= K'_2 / K'_1; Resolution (R)=$V_2-V_1/\frac{1}{2}(W_2+W_1)$; W= peak width; V=retention volume; V_0= void volume; Capacity factor(K´)= $V-V_0/V_0$; Number of theoretical plates (N)=$16(V/W)^2$.

TABLE 3

Chromatographic Constants of (a) Spheri-5-RP-18 and (b) Spheri-5-ODS HPLC Columns (Kontron Analytic)

(a)	Lutein $\frac{}{\alpha}$	Zeaxanthin	K'	N
Lutein	——	1.129	1.476	2704
Zeaxanthin	1.00_R	——	1.666	3136
(b)	Lutein $\frac{}{\alpha}$	Zeaxanthin	K'	N
Lutein	——	1.133	2.142	4356
Zeaxanthin	1.50_R	——	2.428	5184

Acetonitrile:methanol (85:15) was used as mobile phase and flow rate was 1.8 ml/min. Lutein and zeaxanthin injected in ethanol.

Separation factor (α)=K'_2/K'_1;

Resolution (R)=$V_2-V_1/\frac{1}{2}(W_2+W_1)$, where W=peak width and V=retention volume;

Capacity factor (K')=$V-V_0$, where V_0=void volume;

Number of theoretical plates (N)=$16(V/W)^2$.

in this system, the detection limit is considerably more elevated for the apolar carotenoids, tocopherol, tocopherol acetate and retinyl palmitate. The detection threshold was mainly dependent on the retention behavior of the individual compounds; increased retention times resulted in broader and flatter peaks.

With the appropriate adjustment of the UV-detector wavelength, required by the different wavelength maxima of the various carotenoids, as well as reduction of the volume injected, a more selective detection of the individual components can be achieved.

In order to demonstrate the applicability of the present isocratic NARP systems for carotenoid, retinoid and tocopherol separation to biological materials, we analyzed samples of human and vegetable origin.

Application to Human Serum Samples

In figure 6 can be observed the chromatographic profile of a serum sample obtained using system I and double absorbance channel detection. In figure 6a, with the detection channel set at 313 nm and 280 nm, the peaks correponding to retinol, tocopherol and, as internal standards, retinyl acetate and tocopherol acetate are identified. In figure 6b, with the channel at 450 nm, we determined the most representative carotenoids present in serum: lutein plus zeaxanthin, pre-β-cryptoxanthin, beta-cryptoxanthin, lycopene, alpha-carotene and beta-carotene.

Figure 7 is a chromatogram of human serum obtained with system II, with the detection set at 450 nm. Under these conditions, it is possible to identify and quantify lutein and zeaxanthin separately. With system II it is possible to identify a greater number of xanthophils present in serum than those resolved in system I.

The individual determination of the carotenoids in human serum has generally been focused on the quantification of beta-carotene, and in some cases, alpha-carotene and lycopene as well (11,22,24-26,36-38). In recent years, the study of carotenoids isolated in serum has been extended to beta-cryptoxanthin and lutein/zeaxanthin (23,27,28,33,34,39). The analysis of the carotenoids in serum is complemented in some studies by that of retinol and tocopherol (11,13,27,34, 36), although most reports do not analyze the carotenoids, retinoids and tocopherol jointly.

The mobile phase employed in system I, acetonitrile:dichloromethane:methanol (70:20:10) is described

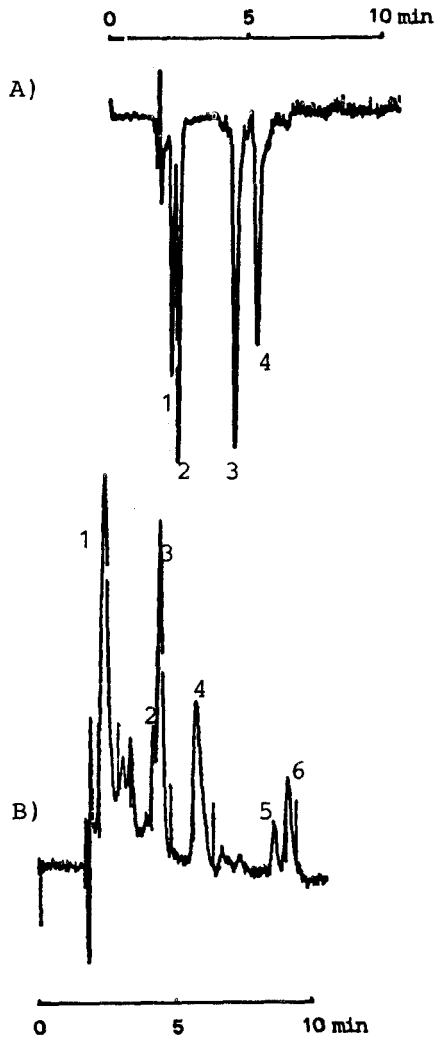

FIGURE 6.- Chromatogram of human serum extract. System I. Peak identification:
A) 1, retinol (313nm, x0.02 AUFS); 2, retinyl acetate (313 nm, x0.02 AUFS); 3, α-tocopherol (280 nm, x0.01 AUFS); 4, tocopheryl acetate (280 nm, x0.01 AUFS).
B) 450 nm, x0.01 AUFS; 1, lutein plus zeaxanthin; 2, pre-β-cryptoxanthin; 3, β-cryptoxanthin; 4, lycopene; 5, α-carotene; 6, β-carotene.

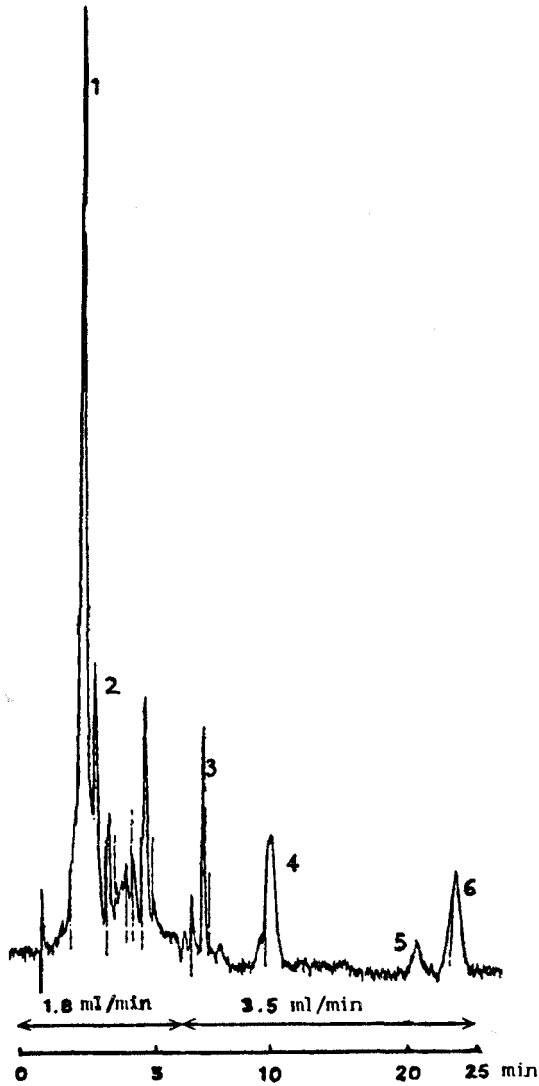

FIGURE 7.- Chromatogram of human serum extract. System II. Detection 450 nm (0.005 AUFS). Peak identification: 1, lutein; 2, zeaxanthin; 3, β-cryptoxanthin; 4, lycopene; 5, α-carotene; 6, β-carotene. Chart speed: 0.3 cm/min.

by Nelis and Leenheer (39) and by Bieri et al. (23), using Zorbax ODS (7 μ) and C-18 columns, respectively, for the determination of the principal carotenoids in serum. Nelis and Leenheer (39) resolve lutein and zeaxanthin, obtaining the analysis of the serum carotenoids in 30 minutes. Bieri et al (23) analyze the same carotenoids in serum in only 13 minutes, but without separating lutein and zeaxanthin. In our chromatographic system I, we are able to resolve simultaneously the main carotenoids to be found in serum, as well as tocopherol, retinol and retinyl palmitate, in a period of 10 minutes. In this system, retinyl acetate, tocopherol acetate and echinenone can be used as internal standards for serum.

After quantification of the sample in system I, the mobile phase is changed to acetonitrile:methanol (85:15), that is system II, which allows a rapid quantification of lutein and zeaxanthin separately.

This method is being applied in the analysis of different carotenoids in serum, as well as retinol, retinyl palmitate and tocopherol. Its sensitivity permits us to study the levels of carotenoids in serum, as well as to detect deficiencies or excesses of vitamins A and E. Hypervitaminosis A results in very high serum levels of retinyl esters (36). Figure 8 shows a chromatogram of the serum of a patient with hypervitaminosis A in which a very elevated level of retinyl palmitate can be observed.

This method is an improvement over that applied until now in our laboratory, which consisted of a C-18 column and mobile phase of methanol:water (95:5) (40), for the determination of vitamins A and E in serum, and with which we have been unable to quantify carote-

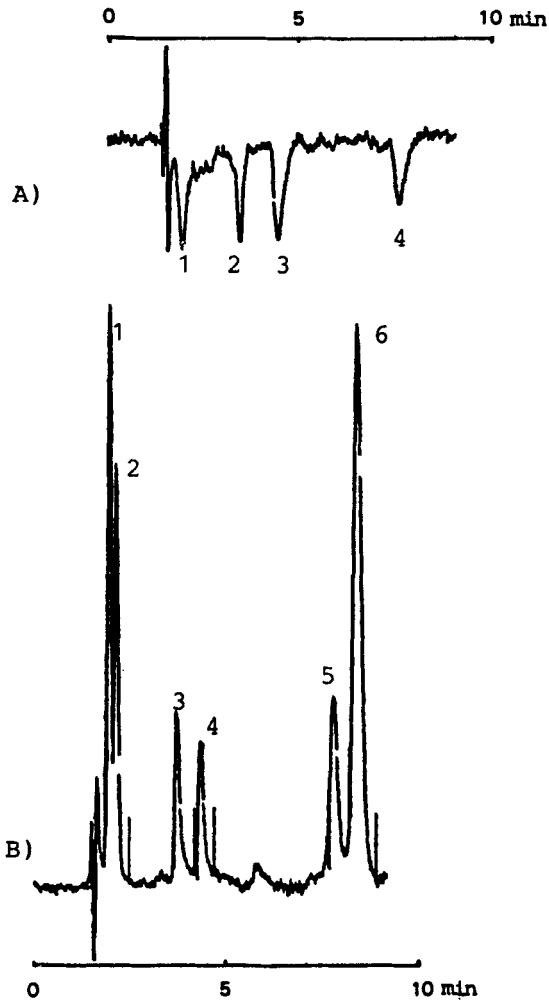

FIGURE 8.- Chromatogram of human serum extract from subject with hypervitaminosis A. System I. Peak identification: A) 1, lutein plus zeaxanthin; 2, β-cryptoxanthin; 3, lycopene; 4, β-carotene. Detection 450 nm, x0.005 AUFS.
B) 1, retinol (313 nm, x0.02 AUFS); 2, retinyl acetate (313 nm, x0.02 AUFS); 3, α-tocopherol (280nm, x0.01 AUFS); 4, tocopheryl acetate (280 nm, x0.01 AUFS); 5, unknown; 6, retinyl palmitate (325 nm, x0.005 AUFS).

noids and retinyl palmitate. Using the method that we describe here, we can determine all these compounds simultaneously; moreover, its sensitivity is greater than that of our old method.

Application to Vegetable Samples

These analytical methods are being applied to the analysis of vegetables. Figure 9 shows the chromatogram of a tomato extract analyzed with both system I and system II. Lutein, lycopene, gamma-carotene and beta-carotene have been identified. The absence of zeaxanthin in this sample has been confirmed by system II (Figure 9b).

As an internal standard in the vegetable samples, we have assayed echinenone, retinyl acetate, retinyl palmitate and tocopherol acetate. Echinenone and tocopherol acetate elute with a retention time that falls between those of the xanthophylls and the carotenes. Thus, they can be employed when they do not coincide with any other peak of the sample being analyzed, and would increase the precision of both the polar and apolar carotenoids. Retinyl palmitate elutes later than beta-carotene, and could serve as a good internal standard for apolar carotenoids in vegetables since it does not interfere with other substances found in this type of sample. In contrast, retinyl acetate does not serve because of its excessively short retention time.

At present, the differential study of carotenoids in food is increasingly more widespread (12,14,16,31) due to the lack of uniformity in the food tables with

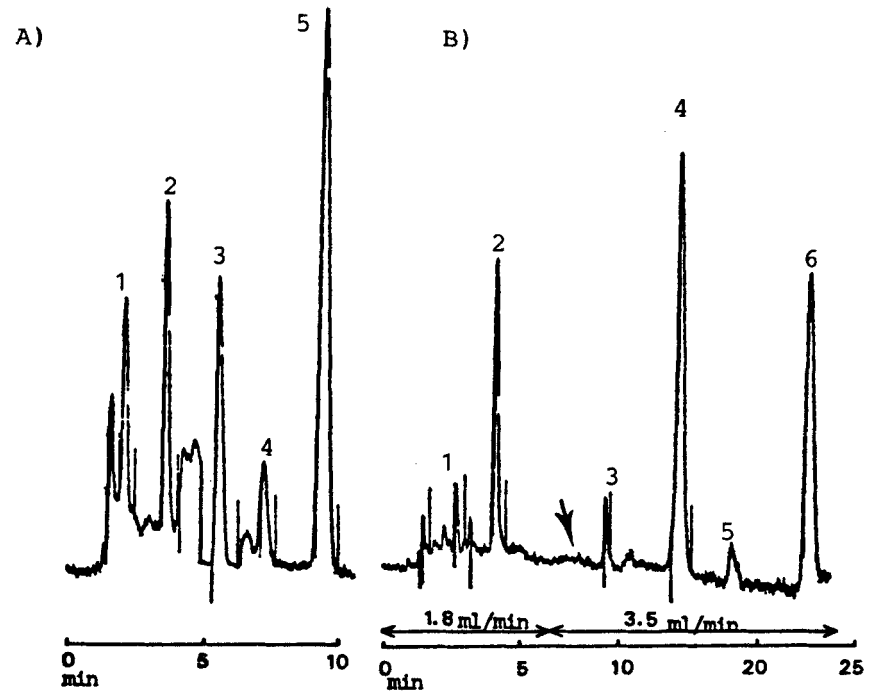

FIGURE 9.- Chromatogram of tomato extract.
A) System I. Peak identification: 1, lutein + zeaxanthin (x0.005 AUFS); 2, unknown; 3, lycopene (x0.1 AUFS); 4, γ -carotene (x0.005 AUFS); 5, β -carotene (x0.005 AUFS).
B) System II. Peak identification: 1, unknown; 2, lutein (0.007 AUFS); 3, unknown; 4, lycopene (0.01 AUFS); 5, γ -carotene (x0.004 AUFS); 6, β-carotene (x0.004 AUFS). Chart speed: 0.5 cm/min and 0.25 cm/min (arrow).

respect to the presentation of data on vitamin A and carotenoids and to the epidemiological studies relating some of these substances to certain pathologies. The application of these methods to vegetables may be of interest, making it possible in the future to include in the food composition tables information about

the intake of specific species of retinoids or carotenoids which to date is not available.

In conclusion, in addition to beta-carotene, several constituents of vegetables and fruits are plausible protective factors against cancer. Although clinical trials of the efficacy of beta-carotene in cancer prevention are underway, it is still necessary and prudent to continue appropriately designed prospective and retrospective studies of the role of vegetables and fruits, as well as carotenoids, tocopherol and other antioxidants, in cancer etiology (41), and to carry out studies of these substances in serum since the hypothesis of an important protective role for these substances remains promising but unproven (19). The methods described would be beneficial to food scientists, cancer researchers and epidemiologists for the accurate estimation of vitamin A activity and the antioxidant capability of food, as well as the levels of these compounds in human serum.

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