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Liquid chromatographic method for the determination of carotenoids, retinoids and tocopherols in human serum and in food

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ABSTRACT

A liquid chromatographic (LC) method has been developed for the quantitative measurement of the six major carotenoids in human serum (lutein, zeaxanthin, β -cryptoxanthin, lycopene, α -carotene, and β -carotene) as well as retinol, retinyl palmitate, α -tocopherol, γ -tocopherol, and δ -tocopherol. Several polar carotenoids, 2',3'-anhydrolutein, α -cryptoxanthin, and geometric isomers of lycopene and β -carotene are also separated. Retinoids and carotenoids are monitored using a programmable ultraviolet-visible detector, while tocopherols are monitored using a fluorescence detector. The method uses a gradient containing acetonitrile, methanol, and ethyl acetate. Ammonium acetate is introduced with the methanol to minimize carotenoid losses on the LC column aggravated by the use of acetonitrile and ethyl acetate. The method is also applicable to the analysis of foods.

INTRODUCTION

Epidemiologists have observed a reduced risk of lung cancer in people who have an elevated intake of fruits and vegetables [1]. Epidemiologic studies also suggest that eating fruits and vegetables may reduce the risk of other cancers as well [1]. Because low serum levels of β -carotene are associated with the subsequent development of

lung cancer, β -carotene may be the protective factor present in the fruits and vegetables [1]. However, the lowered risk of cancer may be due to other carotenoids that are ingested along with the β -carotene, and the importance of these other carotenoids has not been studied adequately.

Six carotenoids (lutein, zeaxanthin, β -cryptoxanthin, lycopene, α -carotene, and β -carotene) reportedly account for more than 90% of the carotenoids present in the serum of American populations [2]. The goal of this study was to separate and measure the six major carotenoids in serum (as well as some of the geometric isomers

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of these carotenoids), retinol, tocopherols, and retinyl palmitate. The reversed-phase liquid chromatographic (LC) method that has been developed allows quantitation of retinol, retinyl palmitate, lutein, zeaxanthin, β -cryptoxanthin, *trans*-lycopene, total lycopene, *trans*- α -carotene, total α -carotene, *trans*- β -carotene, total β -carotene, γ -tocopherol, δ -tocopherol, and α -tocopherol. The method also resolves α -cryptoxanthin, 2',3'-anhydrolutein, and the *cis* isomers of lycopene, α -carotene, and β -carotene, as well as some unidentified polar carotenoids. Most published methods use reversed-phase LC to separate major carotenoids but do not resolve geometric isomers [2-9]. Some workers do report the separation of geometric isomers, usually those of β -carotene, when measuring the major carotenoids [10-12]. Other workers have concentrated on the separation of just β -carotene isomers [13,14] or isomers of both α - and β -carotene [15,16]. To measure serum levels of each major carotenoid accurately, it is important that isomers of that carotenoid not interfere with the measurement of the isomer of interest, otherwise that measurement should be identified as a "total" measurement, *i.e.*, "total β -carotene" as opposed to "*trans*- β -carotene" or the somewhat indefinite " β -carotene." The ideal method would separate all the carotenoids and geometric isomers of interest in the serum as well as the retinoids and tocopherols. Of course, it is difficult to know whether all the isomers are separated; if a method cannot separate two isomers, it is not possible to tell that there are two isomers present merely by looking at a chromatogram that has resulted from the measurement of UV absorbance at a single wavelength.

In work similar to this study, Hess *et al.* [17] separated retinol, the tocopherols, β -cryptoxanthin, lycopene, α -carotene, β -carotene, and some isomers in human serum, and measured them with both UV absorbance and fluorescence; however, they were unable to resolve lutein and zeaxanthin. Using the method described here, we have analyzed standard reference material (SRM) 968a, fat-soluble vitamins in human serum. for the compounds listed above. Although

α -cryptoxanthin, 2',3'-anhydrolutein, and *cis* isomers of lycopene, α -carotene, and β -carotene are resolved from the other components using this method, standards were not available to permit quantitation of these compounds. We have also demonstrated the method's applicability to food by analyzing a mixture of foods.

EXPERIMENTAL^a

Internal standards

Tocol (Esai, Tokyo, Japan) was used as the internal standard for the tocopherols. *trans*- β -Apo-10'-carotenal oxime, the internal standard for the retinoids and carotenoids, was prepared from *trans*- β -apo-10'-carotenal (Hoffman-La-Roche, Basel, Switzerland) [18]. The oxime was purified by passing a concentrated solution through a preparative Vydac 201TP column using 50:50 methanol-acetonitrile and collecting the fraction that contained the *trans*- β -apo-10'-carotenal oxime. The resulting solution was 99.6% pure.

Retinol and carotenoids

Three calibration standards containing known low, intermediate, and high levels (relative to the physiological levels in serum) of lutein (Kemin Industries, Des Moines, IA, USA), zeaxanthin and β -cryptoxanthin (Atomegic Chemetals, Farmingdale, NY, USA), and retinol, α -, and β -carotene (Sigma, St. Louis, MO, USA) were prepared in ethanol with 30 μ g/ml butylated hydroxytoluene (BHT, an antioxidant) added. Structures of retinol, these carotenoids, and *trans*- β -apo-10'-carotenal oxime are shown in Fig. 1. Stock solutions from which standards were prepared were filtered through 45- μ m PTFE syringe filters. Concentrations of these stock solutions were determined spectrophotometrically,

^a Certain commercial equipment, instruments, or materials are identified in this report to specify adequately the experimental procedure. Such identification does not imply recommendation or endorsement by the National Institute of Standards and Technology, nor does it imply that the materials or equipment identified are necessarily the best available for the purpose.

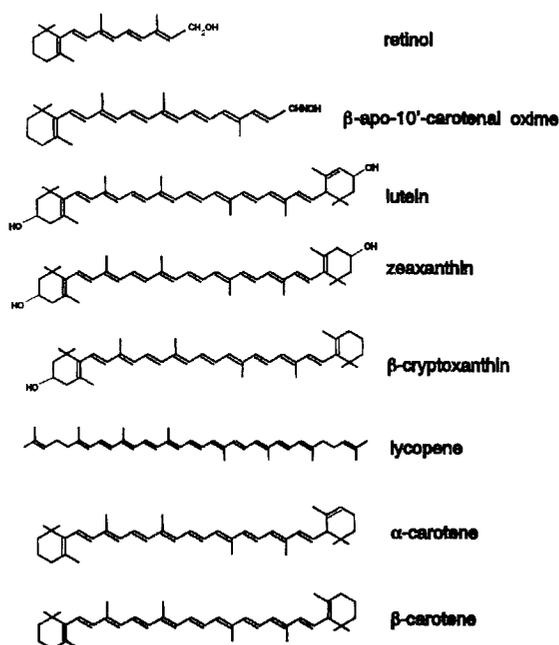


Fig. 1. Structures of retinol, *trans*- β -apo-10'-carotenal oxime, and the carotenoids measured using this method.

and LC analysis of the stock solutions was performed to determine the purity of each solution. Absorptivities and maximum wavelengths used for calibration are shown in Table I. The calibration curve was prepared immediately following preparation of the standards. A 1-ml volume of each calibration solution was combined with

TABLE I
ABSORPTIVITIES AND MAXIMUM WAVELENGTHS USED FOR CALIBRATION

Compound	Absorptivity (dl/g · cm)	λ_{max} (nm)
Retinol	1850	325
Lutein	2765	445
Zeaxanthin	2416	452
β -Cryptoxanthin	2486	452
<i>trans</i> -Lycopene	3450	472
<i>trans</i> - α -carotene	2800	444
<i>trans</i> - β -Carotene	2592	452
Retinyl palmitate	975	325
δ -Tocopherol	91.2	297
γ -Tocopherol	91.4	298
α -Tocopherol	75.8	292

200 μ l of internal standard solution, and the resulting solution was placed in a glass insert in an autosampler vial. The autosampler, which held the samples at 15°C, injected a 20- μ l aliquot of the solution onto the column.

Due to rapid degradation, three lycopene (Sigma) calibration solutions were prepared separately for immediate use. These solutions were also prepared in ethanol with 30 μ g/ml BHT added, and 1 ml of each solution was mixed with 200 μ l of internal standard solution.

Tocopherols and retinyl palmitate

Solutions containing low, intermediate, and high levels of α -, δ -, and γ -tocopherol (Eastman Kodak, Rochester, NY, USA) and retinyl palmitate (Sigma) were prepared as described above. Structures of retinyl palmitate, tocol, α -tocopherol, δ -tocopherol, and γ -tocopherol are shown in Fig. 2.

Serum extracts

SRM 968a, fat-soluble vitamins in human serum (Standard Reference Materials Program, National Institute of Standards and Technology, Gaithersburg, MD, USA), was reconstituted with 1.0 ml of HPLC-grade water. Duplicate

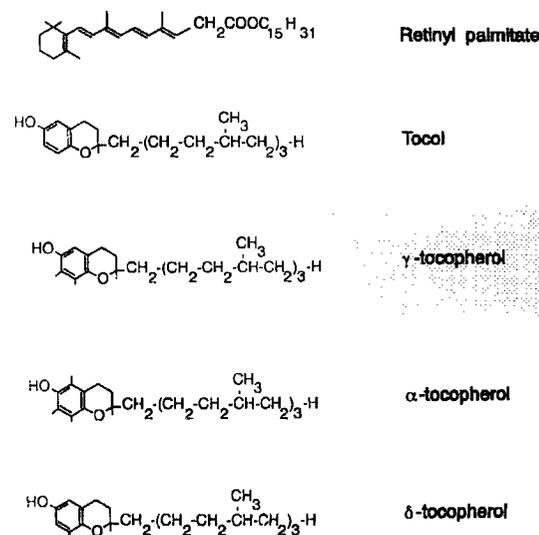


Fig. 2. Structures of retinyl palmitate, tocol, and α -, γ -, and δ -tocopherol.

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200- μ l aliquots of the reconstituted serum were placed in glass test tubes, and serum proteins were denatured with an equal volume of ethanol containing the internal standards and BHT. The samples were mixed on a vortex mixer for 15 s. Hexane (1 ml) was added, and the mixtures were placed on a vortex mixer for 45 s. Then the samples were centrifuged (30 s, \sim 2000 g), and the supernatants were removed. The hexane extraction process was repeated, and the supernatant from this second extraction was combined with that of the first. Extracts were evaporated under a stream of nitrogen and reconstituted in 100 μ l of ethanol containing 30 μ g/ml BHT. The reconstituted extracts were placed in an ultrasonication bath for 30-60 s to facilitate dissolution. The extracts were then transferred to glass inserts that were placed in the autosampler vials.

Quality control

Two sets of serum samples containing low and normal levels of retinol, carotenoids, and tocopherols were run fifteen times over a five-week period to ascertain that the method was in control and to determine the reproducibility of the method. A low and a normal sample were run each day. Peak-area measurements were used for all nine analytes; retinyl palmitate was not measured in these samples.

Food extracts

An extract of a mixture of foods containing apricots, carrots, creamed corn, creamed peas, green beans, orange juice, peaches, spinach, sweet potatoes, tomato paste, and winter squash was analyzed using the method described below to demonstrate the method's applicability to food analysis.

Chromatographic conditions

The method involved two linear gradients and an isocratic step. Solvent A was acetonitrile, solvent B was methanol containing 0.05 M ammonium acetate, and solvent C was ethyl acetate. Each of the three solvents contained 0.05% triethylamine (TEA) for reasons that are discussed below. The first linear gradient began with 98% A-2% B

and went to 75% A-18% B-7% C in 10 min. The second linear gradient ran from this composition to 68% A-25% B-7% C in five min. This composition was held for 10 min longer, then the system was returned to initial conditions (98% A-2% B) over 5 min and re-equilibrated for 10 min.

The precolumn (10 mm \times 4.6 mm I.D., 10 μ m particle size) containing Vydac 201TP polymeric wide-pore C₁₈ (Separations Group, Hesperia, CA, USA) stationary phase and the narrow-pore Bakerbond C₁₈ analytical column (250 mm \times 4.6 mm I.D., 5 μ m particle size, J. T. Baker, Phillipsburg, NJ, USA) were held at 29 \pm 0.5°C by a thermostatted water bath. In previous work, column temperature has been shown to influence selectivity for carotenoids and other compounds with molecule shape differences [19]. The stainless-steel frits throughout the chromatographic system were replaced with biocompatible frits [20]. A programmable UV-Vis detector (Spectroflow 783, ABI, Foster City, CA, USA) with a tungsten lamp was used for measurement of the retinoids and the carotenoids. The wavelength was held at 325 nm to measure retinol and then was changed to 450 nm at 4.5 min to measure the internal standard and the carotenoids; the wavelength was changed back to 325 nm at 22 min to measure retinyl palmitate. A fluorescence spectrometer (LS-4, Perkin-Elmer, Norwalk, CT, USA) was used to measure the tocopherols and tocol using an excitation wavelength of 295 nm and an emission wavelength of 335 nm. Signals from both detectors were recorded simultaneously by the data system (Maxima 820, Waters, Division of Millipore, Milford, MA, USA).

RESULTS AND DISCUSSION

Method development

In a previous paper, we reported on a comparison of carotenoid separations on sixty-five reversed-phase LC columns [20]. Columns were classified as monomeric, polymeric, or intermediate based on their selectivity for a test mixture of polycyclic aromatic hydrocarbons (PAHs), standard reference material 869 [21]. The selectivity test of Sander and Wise classifies phases as

) min. The composition is composed of the system A-2% B) min.

D., 10 μ m polymeric Hesperia, narrow-pore 50 mm \times 4 mm, Baker, Phil-0.5°C by a flow, col-fluence compounds. The stain-atographic stable frits (Spectro-A) with a diameter of the wavelength 1 and then measure the wave-22 min to once spec-walk, CT, herols and of 295 nm. Signals ultaneous-ters, Divi-).

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monomeric, intermediate, or polymeric depending on the elution order of benzo[*a*]pyrene (BaP) and tetrabenzonaphthalene (TBN) ($\alpha_{\text{TBN/BaP}} \leq 1$ for polymeric C_{18} ; $\alpha_{\text{TBN/BaP}} > 1.7$ for monomeric C_{18} phases) [21]. The selectivity of sixty-five columns was evaluated for a mixture of seven carotenoids, using methanol and acetonitrile or these solvents modified with tetrahydrofuran (THF) or ethyl acetate; recovery of the carotenoids from the columns was measured as well. In that study, several conclusions were drawn: under the conditions used, polymeric and some intermediate columns were able to separate lutein and zeaxanthin, but monomeric columns could not; methanol-based solvents provided higher carotenoid recoveries from the column than acetonitrile-based solvents; and if a modifier was used, THF provided slightly better recovery than ethyl acetate and less modifier was required [20].

Column selection

The narrow-pore Bakerbond C_{18} column that was selected for this method is prepared by a polymeric synthesis [22], but was classified as intermediate in the earlier study using the PAH test mixture [21]. A second column, while nominally the same as the column evaluated in the sixty-five-column comparison study, was classified as polymeric, with an $\alpha_{\text{TBN/BaP}}$ of 0.9. The earlier intermediate column ($\alpha_{\text{TBN/BaP}} = 1.27$) was one of the few intermediate columns able to resolve lutein and zeaxanthin in our column comparison study, but its recovery of the carotenoid mixture used in that study was low (75% recovery using 100% methanol as the mobile phase, 66% recovery using 5% THF-95% acetonitrile). These recoveries, which were unacceptable, did not present a problem when recovery was improved by adding ammonium acetate and TEA to the mobile phase.

Solvent selection

Results of the column comparison study indicated that carotenoid recovery was better using THF rather than ethyl acetate and also better using methanol rather than acetonitrile. However, to achieve the desired separation with this col-

umn, it was necessary to use an acetonitrile-based solvent despite the observations described above. We were unable to obtain an acceptable separation using various combinations of THF, methanol, and acetonitrile, and instead developed a method using ethyl acetate, methanol, and acetonitrile.

Acid contamination in chlorinated solvents has been blamed for carotenoid losses [23]; because hydrolysis of ethyl acetate produces acetic acid, acidity may have caused the losses observed when ethyl acetate was used in the column comparison study. Ammonium acetate, which was necessary as a modifier, is poorly soluble in ethyl acetate and in acetonitrile. The addition of 0.05 *M* ammonium acetate to the methanol alone provided sufficient buffer capacity (assuming that acidity is the factor critical to recovery) so that recoveries were high even though ethyl acetate and acetonitrile were used. Although initial separations in an earlier iteration of the method were fine after the column was flushed with buffer, carotenoid recovery gradually decreased without the addition of ammonium acetate to the mobile phase itself. When a mixture of lutein, zeaxanthin, β -cryptoxanthin, lycopene, α -carotene, β -carotene, and *trans*- β -apo-8'-carotenal (an earlier internal standard) was chromatographed using the earlier method without the addition of ammonium acetate, recovery was only 75%. Using the same separation procedure with the addition of ammonium acetate, recovery of that carotenoid mixture was 92%.

Kamber and Pfander [24] found it necessary to add *N,N*-diisopropylethylamine to silica columns to obtain good carotenoid recovery. *N,N*-Diisopropylethylamine is a nucleophilic base; in the absence of this compound, we examined TEA. TEA added to each of the three solvents (acetonitrile, methanol containing 0.05 *M* ammonium acetate, and ethyl acetate) improved recovery. Using the method described in this paper with 0.1% TEA added to each of the three solvents, carotenoid recovery was 101%. When 0.1% TEA was added to the solvents, and the methanol did not contain ammonium acetate, recovery was only 89%. TEA behaves as a strong

modifier, and reduces retention times such that several peaks were no longer resolved when a TEA concentration of 0.1% was used. Thus, as a compromise, this method uses 0.05% TEA in each of the solvents and 0.05 M ammonium acetate in the methanol; recovery is 94%, and the necessary peaks are resolved.

Chromatograms for the analysis of an extract of human serum using this method are shown in

Fig. 3; all analytes were naturally occurring except for retinyl palmitate, which was spiked into the serum. Resolution of lutein and zeaxanthin is superior to most published methods, β -cryptoxanthin is well resolved from lycopene and its isomers, and the *cis/trans* isomers of β -carotene are resolved, although the *cis* isomers are not baseline-resolved. The method's applicability to food separations is demonstrated in Fig. 4. In this fig-

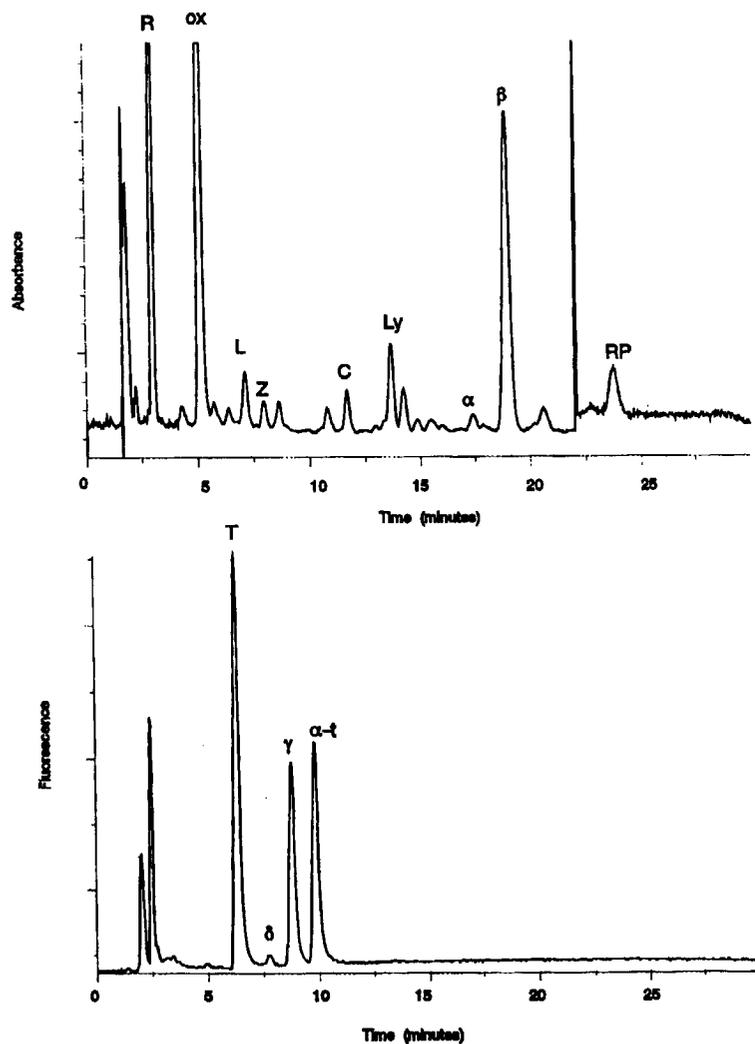


Fig. 3. Chromatogram from the reversed-phase LC separation of retinol (R, 0.734 $\mu\text{g/ml}$), *trans*- β -apo-10'-carotenal oxime (ox), lutein (L, 0.110 $\mu\text{g/ml}$), zeaxanthin (Z, 0.051 $\mu\text{g/ml}$), β -cryptoxanthin (C, 0.060 $\mu\text{g/ml}$), *trans*-lycopene (Ly, 0.124 $\mu\text{g/ml}$), α -carotene (α , 0.022 $\mu\text{g/ml}$), *trans*- β -carotene (β , 0.567 $\mu\text{g/ml}$), and retinyl palmitate (RP, spiked, 0.297 $\mu\text{g/ml}$) in human serum using absorbance detection. Chromatogram of tocol (T), δ -tocopherol (δ , 0.084 $\mu\text{g/ml}$), γ -tocopherol (γ , 1.94 $\mu\text{g/ml}$), and α -tocopherol (α -t, 5.69 $\mu\text{g/ml}$) using fluorescence detection.

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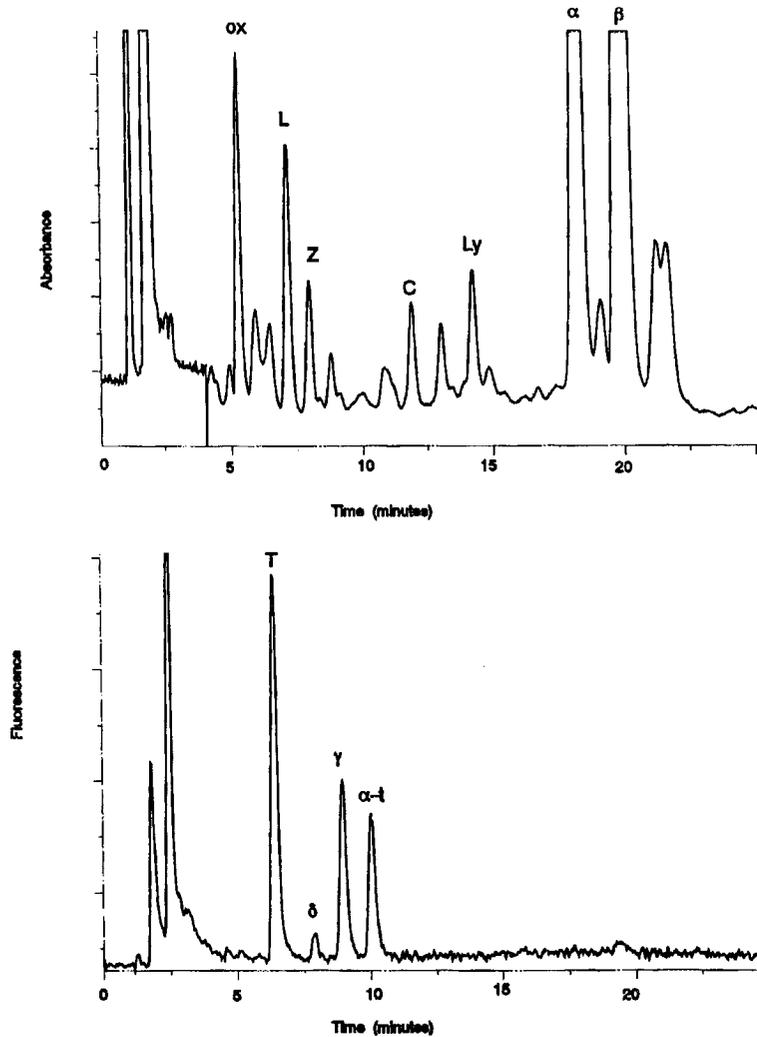


Fig. 4. Chromatogram from the reversed-phase LC separation of retinol, carotenoids, and tocopherols in a mixture of apricots, carrots, creamed corn, creamed peas, green beans, orange juice, peaches, spinach, sweet potatoes, tomato paste, and winter squash. Labels are the same as those for Fig. 3.

ure the chromatograms from the analysis of an extract of the mixture of foods listed in the Experimental section contain all the major peaks that are present in serum as well as additional unidentified peaks. The resolution of the *cis/trans* isomers of α - and β -carotene is more apparent in this chromatogram because of the increased levels of these isomers relative to those in the serum. (The *cis* isomers of α - and β -carotene follow the *trans* compounds, which are labelled, in Figs. 3 and 4.)

Internal standard selection

During the analysis of serum samples other than the SRM, our first choice of internal standard for retinol and the carotenoids, *trans*- β -apo-8'-carotenal, co-eluted with polar carotenoids that were present in some of those samples. Thus, it was necessary to investigate different internal standards. *trans*- β -Apo-10'-carotenal eluted with an appropriate retention time, but only 65% of it was recovered from the column. Using an earlier method that did not employ TEA, the

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 $\mu\text{g/ml}$) using

TABLE II

RESULTS OF THE ANALYSIS OF SRM 968a, FAT-SOLUBLE VITAMINS IN HUMAN SERUM

Mean values were determined from the twelve values that resulted from analysis of two extracts from each of six bottles at each level. Uncertainties (values in parentheses) are one standard deviation from the mean. Uncertainties on certified values represent a 95% confidence interval.

Compound	Results ($\mu\text{g/ml}$)	Certified/reference values ($\mu\text{g/ml}$)	Compound	Results ($\mu\text{g/ml}$)	Certified/reference values ($\mu\text{g/ml}$)
<i>Low level</i>					
Retinol	.199 (0.008)	0.184 \pm 0.016	<i>trans</i> - α -Carotene	0.043 (0.003)	
Lutein	0.035 (0.002)	0.03	Total β -carotene	0.855 (0.056)	0.982 \pm 0.082
Zeaxanthin	0.013 (0.001)	0.02	<i>trans</i> - β -Carotene	0.770 (0.048)	0.9
β -Cryptoxanthin	0.019 (0.002)	0.02	Retinyl palmitate	ND	
Total lycopene	0.160 (0.019)	0.2	δ -Tocopherol	0.112 (0.030)	
<i>trans</i> -Lycopene	0.066 (0.006)	0.07	γ -Tocopherol	2.66 (0.05)	2.8
Total α -carotene	0.022 (0.003)	0.02	α -Tocopherol	10.36 (0.25)	10.40 \pm 0.26
<i>trans</i> - α -Carotene	0.012 (0.001)		<i>High level</i>		
Total β -carotene	0.231 (0.017)	0.264 \pm 0.079	Retinol	0.655 (0.019)	0.648 \pm 0.026
<i>trans</i> - β -Carotene	0.200 (0.013)	0.2	Lutein	0.072 (0.005)	0.08
Retinyl palmitate	ND ^a		Zeaxanthin	0.027 (0.002)	0.03
δ -Tocopherol	0.054 (0.011)		β -Cryptoxanthin	0.038 (0.002)	0.05
γ -Tocopherol	0.801 (0.059)	0.8	Total lycopene	0.440 (0.022)	0.4
α -Tocopherol	4.61 (0.38)	4.44 \pm 0.22	<i>trans</i> -Lycopene	0.151 (0.008)	0.2
<i>Medium level</i>					
Retinol	0.516 (0.013)	0.495 \pm 0.021	Total α -carotene	0.128 (0.007)	0.1
Lutein	0.092 (0.006)	0.09	<i>trans</i> - α -Carotene	0.080 (0.006)	
Zeaxanthin	0.037 (0.003)	0.04	Total β -carotene	2.22 (0.11)	2.65 \pm 0.22
β -Cryptoxanthin	0.057 (0.005)	0.06	<i>trans</i> - β -Carotene	1.92 (0.10)	2.4
Total lycopene	0.461 (0.022)	0.4	Retinyl palmitate	0.022 (0.010)	
<i>trans</i> -Lycopene	0.179 (0.011)	0.2	δ -Tocopherol	0.163 (0.036)	
Total α -carotene	0.066 (0.004)	0.05	γ -Tocopherol	3.70 (0.09)	3.9
			α -Tocopherol	16.04 (0.59)	16.19 \pm 0.56

^a ND = not detected; analyte not present or present at levels below the limit of detection.

addition of a small amount of formaldehyde [500 μl of a 30% (w/v) formaldehyde solution per l of solvent] to each of the three solvents improved recovery of *trans*- β -apo-10'-carotenal (>90% recovery). But since recovery of this internal standard might vary over time, we converted the *trans*- β -apo-10'-carotenal to *trans*- β -apo-10'-carotenal oxime, which has approximately the same retention time as its aldehyde, is quite stable, and does not have a recovery problem. Thus, formaldehyde is no longer being added to the solvents. Although we did not observe loss of β -apo-8'-carotenal in our earlier studies, it is probable that this aldehyde would be subject to the same losses as β -apo-10'-carotenal. For this reason the free

carotenals are not recommended for use as internal standards.

Analysis of serum samples

Results for the analysis of SRM 968a are shown in Table II. Certified values are provided for retinol, total β -carotene, and α -tocopherol in SRM 968a; reference values are provided for lutein, zeaxanthin, β -cryptoxanthin, *trans*-lycopene, total lycopene, α -carotene, *trans*- β -carotene, and γ -tocopherol. Certified values were determined by two NIST analysts using different methods and seven collaborating laboratories using different column-mobile phase combinations. Reference values were determined by a lim-

TABLE III
MEAN STANDARD DEVIATION, AND RELATIVE STANDARD DEVIATION FOR TWO QUALITY CONTROL SERUM SAMPLES MEASURED FIFTEEN TIMES OVER A FIVE-WEEK PERIOD

Compound	Mean ($\mu\text{g/ml}$)	S.D. ($\mu\text{g/ml}$)	R.S.D. (%)
<i>Normal quality control</i>			
Retinol	0.474	0.010	2.1
Lutein	0.117	0.005	4.0
Zeaxanthin	0.039	0.002	4.9
β -Cryptoxanthin	0.058	0.002	3.5
<i>trans</i> -Lycopene	0.222	0.015	6.8
<i>trans</i> - α -Carotene	0.037	0.001	3.5
<i>trans</i> - β -Carotene	0.164	0.005	3.0
δ -Tocopherol	0.076	0.012	15.2
γ -Tocopherol	1.93	0.07	3.7
α -Tocopherol	8.58	0.32	3.8
<i>Low quality control</i>			
Retinol	0.366	0.011	3.1
Lutein	0.091	0.004	4.9
Zeaxanthin	0.030	0.002	7.5
β -Cryptoxanthin	0.044	0.002	3.8
<i>trans</i> -Lycopene	0.167	0.006	3.8
<i>trans</i> - α -Carotene	0.028	0.001	5.3
<i>trans</i> - β -Carotene	0.128	0.004	3.2
δ -Tocopherol	0.059	0.008	14.0
γ -Tocopherol	1.49	0.04	2.4
α -Tocopherol	6.64	0.21	3.2

ited number of analyses and are provided by NIST for information only. (The method described in this paper was not used to assign values to the SRM.) Results show good agreement with reference and certified values for retinol, the tocopherols, and most of the carotenoids. Total β -carotene results obtained using this method are somewhat low for the medium- and high-level SRM when compared to the certified values, but are comparable to results obtained when the SRM was distributed to 45 laboratories involved in a round robin study. As reported in the SRM Certificate of Analysis, the medium- and high-level total β -carotene concentrations determined in the round robin were 0.89 ± 0.12 and 2.34 ± 0.34 mg/l, respectively.

Results for the analysis of the two quality con-

trol sera are shown in Table III. Means are provided to show the levels at which the given relative standard deviations (R.S.D.s) were attained. Most of the analytes had R.S.D.s in the 3-5% range. The R.S.D. for δ -tocopherol is significantly higher due to the very low concentrations present in the serum (about two orders of magnitude lower than α -tocopherol). The R.S.D. for the low-concentration zeaxanthin is also somewhat high, probably because of the relatively low concentration in this sample.

Dissolution of extract

Dissolution of serum extracts is a critical step in the quantitative analysis of carotenoids in serum. The carotenoid concentrations are frequently near the detection limit. The carotenoids and lipid residue are poorly soluble in polar organic solvents, yet the extract must be completely dissolved in a solvent miscible with the mobile phase and an adequate volume of the solvent injected without creating chromatographic artifacts. Dissolution can be facilitated by using ultrasonic agitation. Ideally the extract should be dissolved in the initial mobile phase or a slightly weaker solvent to focus the sample at the head of the column. We compared the effects on quantitation of dissolution in different solvents. After following the usual hexane extraction procedure, dissolution of the dried extract was compared in ethanol and in several other solvents. When 15:85 acetone-acetonitrile, methanol, a 20:80 methylene chloride-methanol, and 20:80 methylene chloride-acetonitrile were added to the dried extract, precipitates formed, and low recoveries were observed. Ethyl acetate dissolved the extract with no difference in recovery when compared to ethanol. Because of the increased solubility of lipids in ethyl acetate, we are currently reconstituting extracts in 50:50 ethyl acetate-ethanol, with 30 $\mu\text{g/ml}$ BHT. It is important to note that peak broadening may become a problem when more than 20 μl are injected into the predominantly acetonitrile mobile phase.

Residual acid and metal activity

The improved recovery resulting from the use

of ammonium acetate and TEA is not limited to the analytical column used in the method described here. During the development of this method, we observed that exposure to minute quantities of trifluoroacetic acid caused the column to become unsuitable for carotenoid separations; previous carotenoid separations on the column were acceptable, but no peaks were obtained after the column had been exposed to traces of trifluoroacetic acid. Treatment with 50:50 methanol-0.01 M ammonium acetate for 45 min restored the column to its initial carotenoid-separating state. The column could then be converted back and forth from "bad" to "good" at will by injecting dilute trifluoroacetic acid and washing the column with solvent containing ammonium acetate.

Following this observation, five columns that had provided poor recovery in our previous sixty-five-column evaluation were flushed with 0.05 M ammonium acetate in methanol at 1 ml/min for 15 min, then equilibrated for 15 min with THF-acetonitrile eluent pumped at 1 ml/min. After this buffer treatment, recovery improved on all five columns. Some columns showed greater improvement than others. Table IV shows the initial recoveries and recoveries following the buffer treatment. Mean improvement in recovery

across this group of five columns was 40%. Thus, other recoveries in the earlier study may have improved if the columns had first been flushed with an ammonium acetate buffer.

After observing that TEA further improved recovery, these same five columns were again flushed for 15 min with 0.05 M ammonium acetate in methanol. Columns were equilibrated for 15 min with 5:95 THF-acetonitrile containing 0.05% TEA, and carotenoid recovery was measured using this same solvent mixture. Results of this study are also included in Table IV. Recovery on all five columns improved dramatically from initial recoveries. Recovery for the fifth column is ">87%" because not all the peaks had eluted during the 25-min run time using the mobile phase composed of 15:85 THF-acetonitrile containing 0.05% TEA. Reproducibility of recovery measurements in the column comparison study was $\pm 6\%$; recoveries in Table IV that are greater than 100% are within the limits of the method.

These same five columns that had provided poor recovery in the sixty-five-column study were also tested for trace metal activity. Acetylacetonone is used as an indicator of metal activity: the sharper the acetylacetonone peak, the less metal activity present [25]. Of the five columns tested, the one with the highest initial recovery had the least metal activity. Columns 4 and 5, two columns from the same manufacturer, exhibited high metal activity. Columns 2 and 3, also from one manufacturer, exhibited similar metal activity, but less than columns 4 and 5. Although adding TEA to the mobile phase improved recovery on these five columns, it is not likely that TEA is functioning as a chelating agent. However, because of its nucleophilic character, TEA may interact with positively charged metal species that may be present in the silica. The use of nucleophilic bases has been previously reported to improve carotenoid recovery on normal-phase LC columns [24,26]. Liew *et al.* [27], in a study of the role of metal ions in bleaching clays, found that Fe^{3+} , Mg^{2+} , and Ca^{2+} play a significant role in the clay's ability to adsorb β -carotene. They suggested that the β -carotene is adsorbed directly to the

TABLE IV
RECOVERY OF A CAROTENOID MIXTURE ON SELECTED COLUMNS BEFORE AND AFTER TREATMENT WITH 0.05 M AMMONIUM ACETATE IN METHANOL WITH AND WITHOUT THE ADDITION OF 0.05% TEA TO AN ACETONITRILE-BASED MOBILE PHASE

Column	Recovery (%)		
	Before treatment	After treatment; no TEA	After treatment; 0.05% TEA in mobile phase
1	48	74	102
2	1	30	99
3	7	87	106
4	15	69	104
5	3	12	>87

40%. Thus, they have im-

proved recovery were again onium ac-

lbrated for containing y was mea- Results of IV. Recov-ramatically he fifth col-peaks had ng the mo-acetonitrile ility of re-comparison IV that are nits of the d provided study were etylacetone ctivity: the is metal ac- tested, the ad the least o columns d high met-n one man-tivity, but dding TEA ry on these is function-cause of its teract with may be pre-hilic bases ove carote- columns the role of that Fe³⁺, role in the ey suggest-ctly to the

ferric ion, in particular, and then undergoes further reaction [27]. At the same time, they believe that the presence of metal ions causes the dissociation of water to form metal hydroxides and protons, which also cause carotenoid degradation [27]. While there is very little water in our system (<1% from the ammonium hydroxide and the acetic acid used to prepare the ammonium acetate), the improvement of carotenoid recovery with the addition of ammonium acetate (a buffer) and TEA (a base) in our study supports their conclusions.

CONCLUSIONS

In this paper, an LC method is described for the measurement of carotenoids, retinol, retinyl palmitate, and the tocopherols in serum and in food. Retinol, retinyl palmitate, and the carotenoids are separated and measured using a programmable UV-Vis detector. The tocopherols are measured simultaneously using a fluorescence detector in series. Apo-carotenals used as internal standards in our initial methods are not acceptable for use in the analysis of all serum samples because of variable recovery and co-elution with serum carotenoids that are more polar than lutein. Because of this, we have examined several other internal standards, and have selected *trans*-β-apo-10'-carotenal oxime.

Using several different columns, we have also demonstrated that by flushing with a mixture of ammonium acetate and methanol, columns that provide little or no recovery of carotenoids can be improved. TEA added to the solvents also improves carotenoid recovery. The ammonium acetate and the TEA appear to be performing two different functions, since the addition of one without the other did not improve recovery as much as the addition of both in studies with the analytical column used with this method.

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