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Journal of Chromatography B, 678 (1996) 187–195

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Liquid chromatographic determination of carotenoids in human serum using an engineered C_{30} and a C_{18} stationary phase

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Received 21 August 1995; revised 31 October 1995; accepted 1 November 1995

Abstract

A C_{30} stationary phase was specifically engineered for carotenoid separations, and carotenoid measurements using this column are compared with those obtained using a somewhat more conventional C_{18} column. Both methods were used to contribute measurements for the certification of carotenoids in Standard Reference Material 968b, Fat-Soluble Vitamins and Cholesterol in Human Serum. Analytes were extracted from the serum into hexane. Measurements on the C_{18} column were made using a gradient of acetonitrile, methanol, and ethyl acetate, which is described in detail elsewhere. Measurements on the C_{30} column were made using a gradient of water, methanol, and methyl *tert.*-butyl ether.

Keywords: Carotenoids

1. Introduction

Carotenoids constitute a group of compounds that continue to be studied for their possible ability to reduce the risk of developing certain types of cancer, and β-carotene has received the most attention in this regard [1]. 'β-Carotene', however, can occur as a variable mixture of *trans*-β-carotene and several mono- and di-*cis* isomers. These *cis* isomers have less provitamin A activity than *trans*-β-carotene, therefore accurate determination of the *trans* form in the presence of *cis* isomers is nutritionally important. AOAC International continues to recognize the need for reliable methods for the measurement of *cis* and *trans* isomers of β-carotene [2].

Cis isomers may have cancer-preventive activity; however, lack of good measurement technology hinders the acquisition of epidemiological data for these compounds. Workers have separated some β-carotene isomers in serum samples; these methods frequently separate *cis* and *trans* isomers, but may not resolve all *cis* isomers from each other [3–8]. Wide-pore polymeric C_{18} columns have been shown to separate geometric isomers of β-carotene [9] and of lutein, lycopene, α-carotene, and β-carotene [10]. Calcium hydroxide and alumina columns are also able to separate the β-carotene isomers [11,12], but these columns are not commercially available and do not separate other carotenoids present in serum [7].

In comparisons of carotenoid selectivity involving dozens of columns, polymerically modified stationary phases [13] have been shown to provide better

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SSDI 0378-4347(95)00494-7

selectivity than monomeric and 'intermediate' columns using liquid chromatography (LC) [14] and supercritical fluid chromatography [15]. Matus and Ohmacht studied the influence of endcapping procedures on column selectivity for xanthophylls, and concluded that better separations of certain polar carotenoids were possible with non-endcapped polymeric C_{18} columns [16]. In 1994, Sander et al. reported on the design of a stationary phase in which various stationary-phase parameters were manipulated to optimize carotenoid separations [17]; polymeric, non-endcapped stationary phases with long carbon chains were found to provide the best overall selectivity for polar and non-polar carotenoids. A column exhibiting high absolute retention and enhanced shape recognition is required for optimal carotenoid separations because of the range of polarities of the carotenoids and their very similar structures. An ideal column would be based on a silica with high surface area and wide pores; however, because these properties are somewhat mutually exclusive, silica with intermediate surface area and pore sizes was selected in the design of the carotenoid column. The enhanced selectivity toward geometric isomers exhibited by this column has been attributed in part to the relative dimensions of the stationary phase. The thickness of the polymeric C_{30} phase has been estimated to be $\geq$25 Å, which is comparable to the dimensions of some carotenoids. By comparison, C_{18} stationary phases range in thickness from ~17 to 21 Å, and it can be envisioned that less complete interactions provide insufficient basis for separation. The ability of the C_{30} column to separate geometric isomers of the major serum carotenoids has been demonstrated using hexane solutions of carotenoids that have been photoisomerized using iodine and ambient laboratory light [18,19].

This paper reports the use of the C_{30} column for quantification of carotenoids in serum-based samples and its application as part of the certification of carotenoid concentrations in Standard Reference Material (SRM) 968b, Fat-Soluble Vitamins and Cholesterol in Human Serum. SRM 968b consists of three vials of lyophilized serum that are designated as 'low', 'middle', and 'high', based on the levels of retinol, α-tocopherol, and β-carotene they contain [20]. This material has been analyzed for six major carotenoids (lutein, zeaxanthin, β-cryptoxanthin, lycopene, *trans*-α-carotene, and *trans*-β-carotene), and several geometric isomers of β-carotene (9-*cis*-, 13-*cis*-, and 15-*cis*-β-carotene) using the C_{30} column. Results from these analyses were used for certification of SRM 968b in conjunction with measurements from two additional LC methods and with results obtained in a round-robin exercise with 40 participating laboratories in which samples of SRM 968b were distributed [21].

2. Experimental[1]

2.1. Instrumentation

Results from two reversed-phase LC columns are compared in this paper: a 4.6 mm × 25 cm, 5-μm particle diameter narrow-pore polymeric C_{18} column with 'intermediate' shape selectivity characteristics [13] (Bakerbond C_{18}; J.T. Baker Inc., Phillipsburg, NJ, USA) and a 4.6 mm × 15 cm, 3-μm polymeric C_{30} column prepared in-house using 20-nm pore diameter silica [17]. The carbon loading for the C_{30} column was determined to be 21.66% (200 m^2/g, 4.24 $\mu mol/m^2$). A polymeric C_{30} 'Carotenoid Column', prepared using the same NIST-developed procedures, is available commercially from YMC, Inc. (Wilmington, NC, USA). The enhanced shape selectivity exhibited by this column toward carotenoid isomers has been attributed to the synergistic effects of polymeric surface modification chemistry, alkyl ligand length, silica substrate configuration (i.e. pore size and surface area) and silanol activity [17]. A 4 cm × 25 cm semi-preparative polymeric C_{18} column (Vydac 201TP, The Separations Group, Hesperia, CA, USA) was used for fractionation of concentrated serum extracts. Samples were analyzed with a liquid chromatograph consisting of a reciprocating piston pump, refrigerated autosampler, and variable-wavelength absorbance detector.

[1] 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.

2.2. Chromatographic conditions

An in-line filter holder containing a 2-μm titanium frit and the C_{30} column with 2-μm Hastalloy C frits were held at 26.6 ± 0.1°C by a thermostatted water bath. In studies using various C_{18} columns, ammonium acetate and triethylamine (TEA) improved carotenoid recoveries from analytical columns [3]; therefore, these modifiers were also added to the solvents used in these analyses. Solvent A was 8% water/92% methanol containing 0.05 mol/l ammonium acetate and 0.05% TEA; solvent B was methyl *tert.*-butyl ether (MTBE). The method consisted of two linear gradients and an isocratic component. The gradient began with 83% A/17% B and went to 59% A/41% B in 29 min. A second linear gradient ran from this composition to 30% A/70% B in 5 min. This composition was held for 4 min longer to allow lycopene to elute; then the system was returned to initial conditions (83% A/17% B) over 5 min and re-equilibrated for 10 min. A programmable UV/visible detector with a tungsten lamp was used for measurement of carotenoid absorbance at 450 nm.

The LC method using the narrow-pore C_{18} column has been previously described [3]. Briefly, the method consisted of two linear gradients and an isocratic component. Solvent A was acetonitrile, solvent B was methanol containing 0.05 mol/l ammonium acetate, and solvent C was ethyl acetate. Each of the three solvents contained 0.05% TEA to enhance carotenoid recovery. The first gradient began with 98% A/2% B and went to 75% A/18% B/7% C in 10 min. A second linear gradient ran from this composition to 68% A/25% B/7% C in 5 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.

2.3. Standards

Three calibration standards containing known low, intermediate, and high levels (relative to the physiological levels in serum) of lutein and zeaxanthin (Hoffmann-LaRoche, Nutley, NJ, USA), β-cryptoxanthin (Atomergic Chemetals, Farmingdale, NY, USA), and *trans*-α- and *trans*-β-carotene (Sigma, St. Louis, MO, USA) were prepared in ethanol with 30 μg/ml butylated hydroxytoluene (BHT, an antioxidant) added.

Stock solutions of each of these individual compounds were stored at −20°C. Xanthophylls were dissolved in ethanol containing 30 μg/ml BHT; carotenes were dissolved in hexane containing 30 μg/ml BHT. Prior to the preparation of calibration solutions, the stock solutions were placed in an ultrasonicating bath for 5 min to re-dissolve crystals that may have formed during storage. Approximately 10 ml of each stock solution were then passed through a 0.45-μm polypropylene syringe filter. The concentrations of these filtered solutions were calculated using Beer's Law and absorbance readings obtained from a spectrophotometer; wavelengths and absorptivities used are provided in Table 1. LC analysis of the filtered solutions was used to determine the purity of each solution at the wavelength at which the spectrophotometric measurements were made, and the concentration was then corrected to reflect this purity. Appropriate volumes of these solutions of known concentration were then combined to prepare the three calibration standards.

Calibration measurements were carried out immediately following preparation of the standards. An aliquot of each calibration solution was combined with *trans*-β-apo-10′-carotenal oxime internal standard solution [22,23], and the resulting solution was placed in a glass insert in an autosampler vial. Samples were held at 15°C in an autosampler, and 20-μl aliquots were injected for each analysis. Peak areas were used for quantitation of all analytes.

Due to lycopene's comparatively rapid degradation, three lycopene (Sigma, St. Louis, MO, USA)

Table 1
Absorptivities and wavelengths used for determination of calibrant concentrations

Compound	Absorptivity (dl/g·cm)	λ_{max} (nm)	Solvent
Lutein	2765	445	Ethanol
Zeaxanthin	2416	452	Ethanol
β-Cryptoxanthin	2486	452	Ethanol
trans-Lycopene	3450	472	Hexane
trans-α-Carotene	2800	444	Hexane
trans-β-Carotene	2592	452	Hexane
9-*cis*-β-Carotene	2550	445	Hexane
13-*cis*-β-Carotene	2090	443	Hexane
15-*cis*-β-Carotene	1820	447	Hexane

calibration solutions were prepared separately for immediate use. Calibration solutions for 9-*cis*-, 13-*cis*-, and 15-*cis*-β-carotene (Hoffmann-LaRoche, Nutley, NJ, USA) were also prepared separately. These calibration solutions were prepared as described above.

2.4. Serum samples

Extraction of serum samples was performed as has been previously described [3]. SRM 968b samples (NIST, Standard Reference Materials Program, Gaithersburg, MD, USA) were selected for analysis according to a stratified random sampling scheme; SRM 968a [24] was run for quality control. The LC method using the narrow-pore C_{18} column was used to assess the homogeneity of the SRM; consequently more samples were analyzed using this method than the one employing the C_{30} column.

2.5. Carotenoid fractionation

Human serum contains carotenoids and isomers for which standards are not commercially available. To determine the retention times of such carotenoids using the C_{30} column, 10 ml of serum were extracted using a scale-up of the procedure described previously [3] to provide 110 μl of a concentrated serum extract. This extract was injected onto a semi-preparative-scale Vydac 201TP polymeric C_{18} column. Analytes were eluted from the column at room temperature using a mobile phase of 90% methanol/10% ethyl acetate pumped at 10.0 ml/min. An attempt was made to decrease selectivity for this separation so that geometric isomers would not be resolved. Seven fractions were collected: (1) lutein and zeaxanthin, (2) 2′3′-anhydrolutein (tentative assignment), (3) α-cryptoxanthin (tentative assignment), (4) β-cryptoxanthin, (5) α-carotene, (6) β-carotene, and (7) lycopene. Approximately 10 ml of hexane and an excess of water (>50 ml) were added to extract the carotenoids from these fractions. The hexane was evaporated under a stream of nitrogen, and the fractions were reconstituted in 100 μl of a 50:50 mixture of ethanol/ethyl acetate containing 30 μg/ml BHT. These extracts were then injected onto the C_{30} column using the chromatographic conditions described above.

3. Results and discussion

As part of the certification process for a natural-matrix SRM, the material is typically analyzed using two or more 'chemically independent' analytical techniques, and the results of these analyses, if in agreement, are used to determine the certified concentrations of the measured analytes. The requirement for using two or more different techniques is based on the assumption that the agreement of results from independent methods implies that the results are unbiased. If results are obtained from only one analytical technique, the concentrations are generally reported as 'non-certified' or 'information' values.

For the certification of carotenoids in serum, we have relied on measurements performed on LC stationary phases with different selectivity (i.e. different relative retention) to provide the necessary independent methods. In the previous certification of carotenoids in serum, measurements were performed using polymeric C_{18} stationary phases on wide-pore and narrow-pore silica, which provide different selectivity for the separation of carotenoids. Only total α-carotene and *trans*- and total β-carotene were measured using the wide-pore C_{18} column. Therefore, results for other serum carotenoids were provided by only one LC method and were presented as non-certified values in SRM 968a. (Most of the laboratories participating in the round-robin exercise do not measure carotenoids other than total lycopene, total α-carotene, and total β-carotene, so inclusion of data from the interlaboratory exercise does not necessarily provide an additional set of independent results.)

The development of the C_{30} column, which was specifically designed for the separation of carotenoid isomers, provided the opportunity for the use of an additional method with very different selectivity for the certification of SRM 968b. An attempt was made to measure all of the major serum carotenoids using the narrow-pore C_{18} column and the polymeric C_{30} column. Results from these two independent methods could then be compared and certified values provided if results were in agreement. In addition, the C_{30} column can provide information about the *cis* isomers of β-carotene that is not provided by the C_{18} column because of co-elution of 13-*cis*- and 15-*cis*-β-carotene.

Carotenoid concentrations were certified in SRM 968b based on the results from NIST's three LC methods using the narrow-pore and wide-pore polymeric C_{18} columns and the polymeric C_{30} column, and the results from the interlaboratory comparison exercise among 40 laboratories. The results obtained for the carotenoids in these certification measurements using the C_{30} and the narrow-pore C_{18} columns are compared and the differences are discussed below. The details concerning the complete certification of fat-soluble vitamins, carotenoids, and cholesterol in SRM 968b are described elsewhere [21].

In Table 2, results for the C_{30} column are compared with results obtained using the narrow-pore C_{18} column for the analysis of SRM 968b. Most results obtained using the C_{30} column agree with results obtained on the C_{18} column. Fig. 1 and Fig. 2 show the chromatograms from the LC separation of a middle-level sample of SRM 968b using the C_{30} and the C_{18} columns, respectively. For the peaks that are identified, the most distinctive differences between the two columns are the resolution of the isomers of β-carotene and the order of elution of the hydrocarbon carotenoids.

Although the C_{30} column exhibits enhanced selectivity toward geometric isomers, this retention behavior can create problems when the isomers co-elute with other analyte peaks, particularly if the presence of a co-eluting peak is not immediately recognized. Standards are not available for many of the carotenoids found in human serum, so retention times cannot be compared to provide even tentative identifications. Because of disagreements between results from the two columns for lycopene and 13-*cis*- and 15-*cis*-β-carotene, fractions of a concentrated extract of the middle level of SRM 968b were collected from a semi-preparative-scale C_{18} column and injected onto the C_{30} column to provide information about possible co-eluting interferences. Results for each carotenoid are discussed individually below and refer to the values in Table 2.

3.1. β-Carotene

Using the C_{30} column, total β-carotene in the serum samples was calculated by summing the results for *trans*-, 9-*cis*-, 13-*cis*-, and 15-*cis*-β-carotene, which were determined using response factors obtained for each of these individual carotenoids. Results for 15-*cis*-β-carotene in the low level are not reported because of the difficulty in integrating this peak, which is not well resolved from an adjacent peak and is barely larger than the noise. Results for both total and *trans*-β-carotene agree with certified and non-certified values in the SRM 968a quality control samples and with the values obtained using the C_{18} column for SRM 968b. Results for 9-*cis*-β-carotene in SRM 968b also agree well between the two columns.

A concern with the SRM 968b data was the disagreement between the sum of the 13-*cis*- and 15-*cis*-β-carotene results from the C_{30} column and the C_{18} column. To understand the source of this disagreement, fractions were collected from a concentrated serum extract to see whether there were co-eluting peaks interfering with the measurement. The fractions collected are shown in Fig. 3. By examining the C_{30} chromatograms of fractions 4 and 6 in Fig. 4, it is apparent that the β-cryptoxanthin fraction contains a peak that co-elutes with the 13-*cis*-β-carotene isomer on the C_{30} column. Such co-elution would create a positive bias in 13-*cis*-β-carotene results. However, correcting the 13-*cis*-β-carotene concentration for the area resulting from the co-elution of this co-eluting peak does not reduce the sum of 13+15-*cis*-β-carotene sufficiently to equal the sum on the C_{18} column. Instead, in the middle level, the corrected 13-*cis*-β-carotene peak alone (0.031 μg/ml) is comparable with the 0.030 μg/ml obtained for 13+15-*cis*-β-carotene obtained using the C_{18} column, which implies that the 15-*cis*-β-carotene result obtained using the C_{30} column may be biased high as well, possibly by co-elution with an α-carotene isomer. The co-elution of peaks with the *cis* isomers of β-carotene also has an effect on the result for total β-carotene (the sum of *trans*-, 9-*cis*-, 13-*cis*-, and 15-*cis*-β-carotene); however, these peaks are small relative to the *trans*-β-carotene in the samples, and total β-carotene results between the two columns are in agreement.

3.2. α-Carotene

Emenhiser et al. have unequivocally identified *cis* isomers of α-carotene in an isomerized solution [19]. The identities of α-carotene isomers present in

Table 2
Mean results (μg/ml) for the analysis of SRM 968b, fat-soluble vitamins and cholesterol in human serum using the C_{30} column ($n = 8$) compared with values obtained using the C_{18} column ($n = 48$)

	C_{30}	C_{18}
Low level		
Lutein	0.0592±0.0015	0.0583±0.0015
Zeaxanthin	0.0181±0.0007	0.031[a]±0.002
β-Cryptoxanthin	0.0196±0.0012	0.0243±0.0013
trans-Lycopene	0.1070[b]±0.0033	0.0899±0.0026
Total lycopene	0.1491±0.0039	0.235[c]±0.015
trans-α-Carotene	0.0146±0.0012	0.0183±0.0010
Total α-carotene		0.0251±0.0077
trans-β-Carotene	0.2131±0.0096	0.231±0.014
9-*cis*-β-Carotene	0.0075±0.0019	0.0078±0.0011
13-*cis*-β-Carotene	0.0182[d]±0.0073	Not measured
15-*cis*-β-Carotene	Not measured	Not measured
13+15-*cis*-β-Carotene		0.0126±0.0006
Total β-carotene	0.239±0.011	0.2547±0.0058
Middle level		
Lutein	0.0576±0.0024	0.0632±0.0013
Zeaxanthin	0.0221±0.0030	0.042[a]±0.001
β-Cryptoxanthin	0.0271±0.0033	0.0321±0.0013
trans-Lycopene	0.1703[b]±0.0032	0.1481±0.0039
Total lycopene	0.2442±0.0073	0.359[c]±0.011
trans-α-Carotene	0.0290±0.0038	0.0284±0.0010
Total α-carotene		0.0433±0.0022
trans-β-Carotene	0.573±0.019	0.585±0.014
9-*cis*-β-Carotene	0.0185±0.0025	0.0167±0.0039
13-*cis*-β-Carotene	0.0395[d]±0.0048	Not measured
15-*cis*-β-Carotene	0.0257±0.0034	Not measured
13+15-*cis*-β-Carotene		0.0305±0.0010
Total β-carotene	0.656±0.024	0.641±0.016
High level		
Lutein	0.0340±0.0019	0.0427±0.0012
Zeaxanthin	0.0123±0.0013	0.032[a]±0.001
β-Cryptoxanthin	0.0257±0.0022	0.0333±0.0020
trans-Lycopene	0.1986[b]±0.0098	0.161±0.036
Total lycopene	0.284±0.013	0.406[c]±0.020
trans-α-Carotene	0.0363±0.0032	0.0373±0.0029
Total α-carotene		0.0618±0.0074
trans-β-Carotene	1.155±0.064	1.16±0.27
9-*cis*-β-Carotene	0.0301±0.0033	0.0274±0.0070
13-*cis*-β-Carotene	0.0676[d]±0.0055	Not measured
15-*cis*-β-Carotene	0.0417±0.0046	Not measured
13+15-*cis*-β-Carotene		0.0595±0.0054
Total β-carotene	1.295±0.069	1.27±0.22

Uncertainties are expanded uncertainties at the 95% confidence level, and include measurement uncertainty within the analytical method but do not include vial-to-vial variability.

[a] Unknown peak co-elutes with zeaxanthin.

[b] Believed to be a combination of *trans*-lycopene and one or more co-eluting *cis* isomers.

[c] Non-lycopene peak may be grouped with 'total lycopene'.

[d] Peak that is believed to be an isomer of β-cryptoxanthin co-elutes with 13-*cis*-β-carotene.

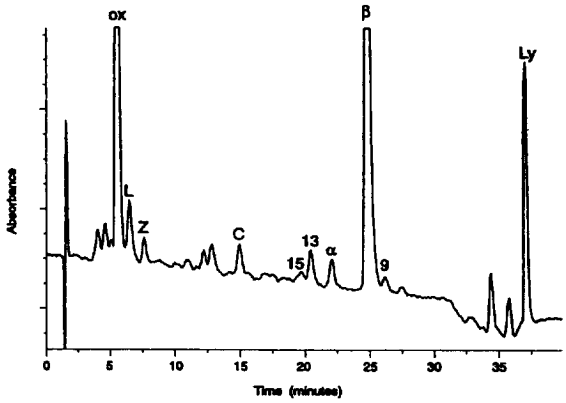

Fig. 1. Chromatogram from reversed-phase LC analysis of the middle level of SRM 968b using the C_{30} column with absorbance detection at 450 nm. Peak identifications: *trans*-β-apo-10′-carotenal oxime (ox), lutein (L), zeaxanthin (Z), β-cryptoxanthin (C), 15-*cis*-β-carotene (15), 13-*cis*-β-carotene (13), α-carotene (α), *trans*-β-carotene (β), 9-*cis*-β-carotene (9) and lycopene (Ly). Chromatographic conditions are described in the text.

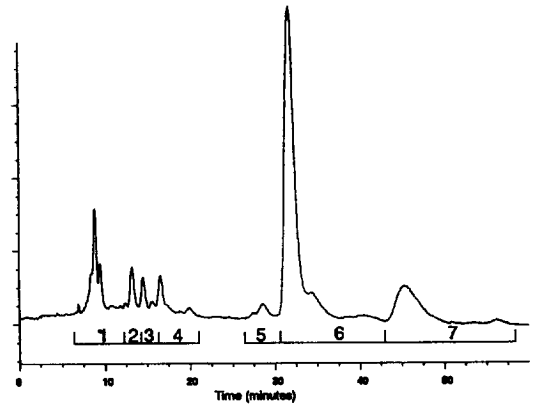

Fig. 3. Chromatogram from semi-preparative-scale LC separation of concentrated extract of the middle level of SRM 968b with absorbance detection at 450 nm. Numbered brackets indicate the seven fractions collected. Chromatographic conditions are described in the text.

serum remain to be determined. Fig. 4 shows the *trans*-α-carotene peak as well as six other smaller peaks collected in fraction 5. Four of these small peaks correspond to identified isomers of β-carotene and are probably β-carotene compounds that were collected with the α-carotene fraction. The remaining small peaks that do not line up with peaks in the other fractions may be *cis* isomers of α-carotene, but these peaks are too small to be quantified in the original serum extracts (Fig. 1), and are not large enough to account for the *cis* isomer concentration

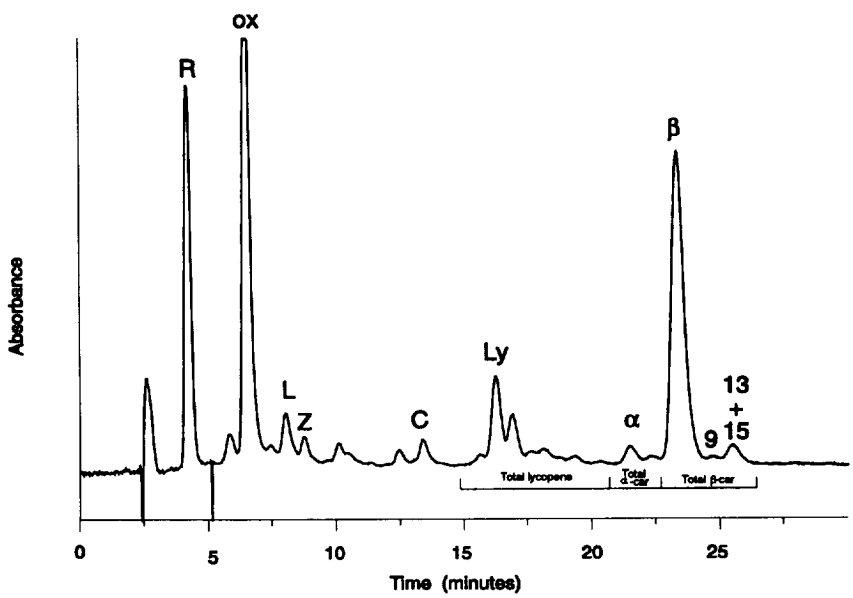

Fig. 2. Chromatogram from reversed-phase LC analysis of the middle level of SRM 968b using the C_{18} column with absorbance detection at 325 and 450 nm. Peak identities are the same as those provided for Fig. 1. Chromatographic conditions are described briefly in the text and in Ref. [3].

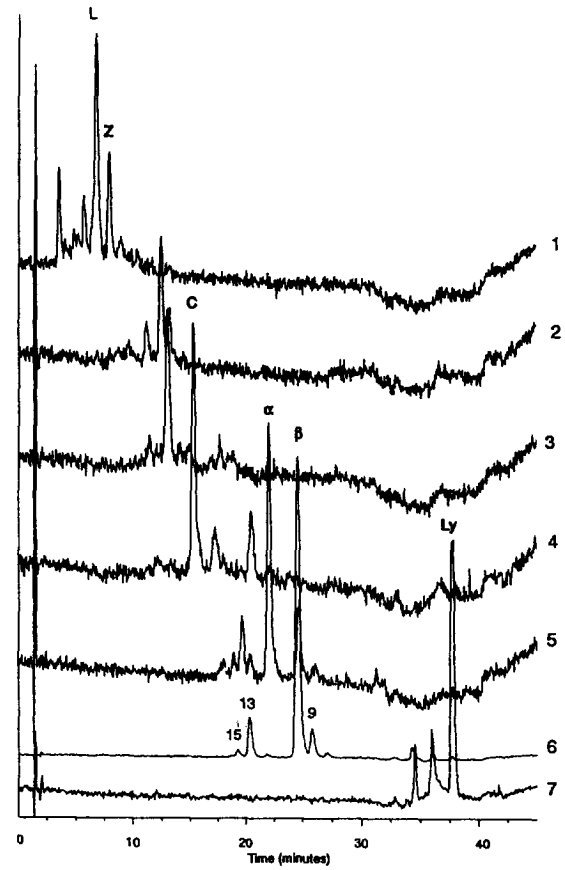

Fig. 4. Chromatograms from reversed-phase LC separation of the fractions shown in Fig. 3 using the C_{30} column. Peaks for which standards are available are identified using the abbreviations shown in Fig. 1. Chromatographic conditions are described in the text.

implied by the difference between the results for total and *trans*-α-carotene using the C_{18} column. Results for *trans*-α-carotene using the C_{30} column agree well with the values obtained using the C_{18} column for SRM 968b.

3.3. Lycopene

The retention times of lycopene isomers using the C_{30} method also had not been previously identified. Given the 15–25% disagreement between the '*trans*-lycopene' values in SRM 968b using the C_{30} column and the C_{18} column, it is apparent that the gradient used with the C_{30} column does not separate *trans*-lycopene from at least one of its isomers. The two isomers that are separated elute before the peak identified as lycopene in fraction 7 of Fig. 4; no additional peaks elute when the method is allowed to run at 30%A/70% B for an additional 20 min. Total lycopene results were calculated for the SRM 968b samples by combining these three lycopene peaks. These total lycopene values are much lower (30–40% disagreement for both SRM 968a and 968b) than the total lycopene values obtained using the C_{18} column, therefore it is possible that some of the peaks combined for 'total lycopene' using the C_{18} column should not be included in that grouping. Neurosporene, ζ-carotene, and/or γ-carotene may be mis-grouped with the 'total lycopene' on the C_{18} column.

3.4. Lutein

Results for lutein using the C_{30} column agree with non-certified values for SRM 968a, and results for SRM 968b using the C_{30} column agree with results obtained using the C_{18} column.

3.5. Zeaxanthin

Results for zeaxanthin in SRM 968b do not agree with results obtained using the C_{18} column because of a peak co-eluting with zeaxanthin on the C_{18} column throughout this study. Values for zeaxanthin in SRM 968a using the C_{30} column, however, are the same as the non-certified values provided.

3.6. β-Cryptoxanthin

Results for β-cryptoxanthin using the C_{30} column are slightly lower than the reference values for SRM 968a and the values obtained using the C_{18} column for SRM 968b. This is probably due to differences in resolution of isomers between the two columns. For example, the value obtained using the C_{30} column may be *trans*-β-cryptoxanthin, and the value obtained on the C_{18} column may include an unresolved *cis* isomer.

4. Conclusions

We have developed and evaluated an LC method for measuring serum carotenoids using a C_{30} column designed specifically for carotenoid analysis. The method has provided good accuracy in the analysis of SRM 968a and was used to provide values for the certification of SRM 968b. Because of the potential for many isomer peaks to be separated by this column, collection of fractions separated by a less selective column and subsequent separation on the C_{30} column can help to determine the location in the chromatogram of isomers for which standards are not commercially available.

Rather than the C_{30} column being superior to the C_{18} column with which it was compared, the two columns provide complementary information. For example, using the mobile-phase gradients described, results are provided for *trans*-lycopene using the C_{18} column and for total lycopene using the C_{30} column. Of course, refinement of the gradient may allow *trans*-lycopene to be measured accurately using the C_{30} column, and identification of the peak that is incorrectly grouped with total lycopene on the C_{18} column would result in an accurate total lycopene measurement. Ideally, such refinements and identifications will result in two independent approaches for the measurement of serum carotenoids.

Acknowledgments

We thank Hoffmann-LaRoche for providing lutein, zeaxanthin, and 9-*cis*- 13-*cis*-, and 15-*cis*-β-carotene, as well as *trans*-β-apo-10′-carotenal for the synthesis of the *trans*-β-apo-10′-carotenal oxime internal standard. We also thank Jörg Kutter (University of Ulm, Germany) for assisting in the preparation of the C_{30} column used in this study.

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