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USE OF REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS FOR THE DETERMINATION OF PROVITAMIN A CAROTENES IN TOMATOES*

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SUMMARY

The usual methods for provitamin A evaluation of foods convert the total pigment amount, determined spectrophotometrically, into vitamin A units. Since the totally inactive lycopene is the major carotenoid in the tomato, such readings result in erroneously high provitamin A values.

In view of the recent development of chemically bonded, reversed-phase, microparticulate packings and their use in high-performance liquid chromatography which combines highly accurate and reproducible resolution with the speed and ease of operation, a new method using such a system was developed to isolate carotenoid pigments from tomato samples. A 15-min column separation was thus achieved, dramatically decreasing the analysis time of the classical open column chromatographic procedures, which often result in unresolved and altered fractions due to long-term exposure to oxygen, light, solvents and sometimes adsorbent.

β-Carotene and lycopene were determined and quantitated in six tomato samples. β-Carotene, 100% vitamin A-active, was expressed in International Units of vitamin A. The newly developed method gives a more reliable evaluation of the fruit potency in vitamin A than the methods of the Association of Official Analytical Chemists currently used for food composition tables.

INTRODUCTION

Differences in the biopotency of carotenoids as vitamin A precursors result from their individual structures. The β-ring present in retinol is essential for their

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activity. β-Carotene, having two such rings, is considered 100% vitamin A-active. α-Carotene is only one half as potent while acylcic carotenes such as lycopene, also naturally present in foods, are totally inactive (Fig. 1). The determination of provitamin A levels in foods hence requires the isolation and accurate quantitation of those carotenes with biological significance. Few of the analytical methods available are suitable for this purpose: open-column chromatography, besides being time-consuming and allowing long-term exposure of carotenoids to oxygen, light, adsorbents and solvents, often fails in resolving the most potent vitamin A precursor, all-*trans*-β-carotene, from its less active geometrical isomer, α-carotene (1); both this method and thin-layer chromatography lack in reproducibility and accurate quantitation[1,2]; gas chromatography provides the latter advantages but cannot be used with the thermally labile carotenoids[3,4].

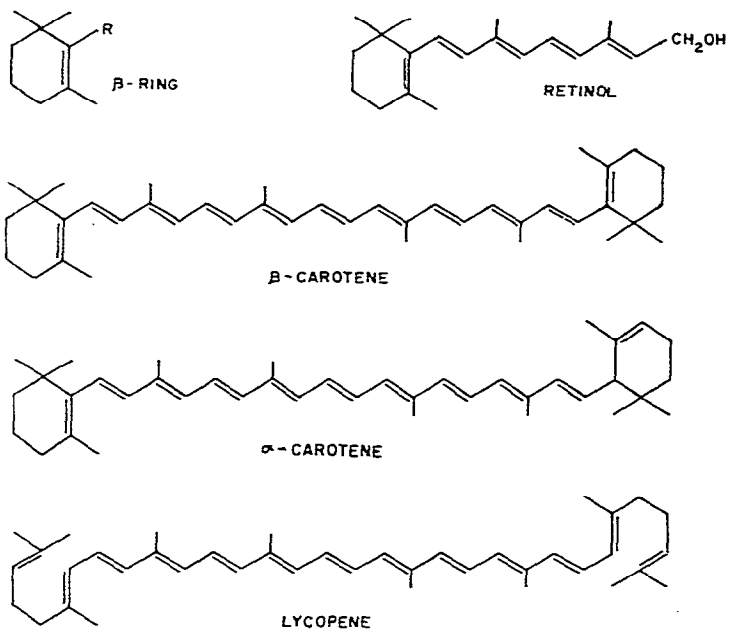

Fig. 1. Importance of β-ring in carotenoid activity as vitamin A precursors.

The advent of high-performance liquid chromatography (HPLC), which made available rapid, reproducible, quantitative and accurate analyses, opened up new possibilities for the study of carotenoids. The use of this technique together with the recently developed microparticle packings has resulted in faster solute distributions between the mobile and stationary phases, hence sharper elution profiles for individual compounds and the increased resolution capacity for a given separation[5].

Although some carotenes could not be separated by HPLC on silica [6,7], a carotenoid mixture was resolved on both magnesium oxide[1] and alumina columns[8] by gradient elution; however, long re-equilibration periods at initial conditions were necessary for subsequent analysis[8]. The isolation of some isoprenoids and polyprenols was also successful on chemically-bonded, reversed-phase packings[9,10]. These non-

polar stationary phases present several advantages over the normal-mode polar adbents: they are neutral to the sample and unaffected by the presence of water or changes in the mobile phase [9,11]. This inherent stability renders them most suitable for routine sample analysis. The separation of carotenes was thus attempted by the use of organic eluting solvents in reversed-phase, and the resolution of β-carotene from other carotenoids was sought for estimation of provitamin A values in tomato samples.

MATERIALS AND METHODS

Standard solutions

All standards were purchased from Sigma (St. Louis, Mo., U.S.A.). Crystalline α-carotene was dissolved in petroleum ether; the resulting solution contained 333.75 μg/ml (0.5216 mmoles/l). Crystalline β-carotene was dissolved in petroleum ether (b.p., 40–60°) the resulting solution contained 154 μg/ml (0.2869 mmoles/l). The concentration of lycopene dissolved in dichloromethane was 166.66 μg/ml (0.3104 mmoles/l, solution I); 1 ml of this solution in 25 ml dichloromethane constituted solution II (0.0124 mmoles/l).

The retention times of lycopene, α- and β-carotene averaged 7.82, 12.4 and 13.2 min, respectively. The peak areas were measured for quantitation.

Liquid chromatograph

A Waters Model 6000A Solvent delivery system (Waters Assoc., Milford, Mass., U.S.A.) was used. This is a reciprocating plunger pump which permits digital selection of a constant flow-rate. Waters U6K injector system allowed loading of the sample at atmospheric pressure and assured accurate injections (no sample loss due to back pressure on the system). A Waters Model 450 Variable Wavelength Detector was set at 470 nm. This wavelength was found most suitable for the simultaneous detection of α-carotene, β-carotene and lycopene.

A SF 770 Spectroflow Monitor variable-wavelength detector equipped with SFA 339 Wavelength drive and MM 700 Memory Module, all from Kratos Inc., Schoeffel Instrument Division (Westwood, N.J., U.S.A.), was used for obtaining stopped-flow visible spectra.

Chromatographic peaks were recorded on a Houston Omniscribe recorder. The following chromatographic columns were used in the course of this study: stainless-steel (30 cm × 3.9 mm I.D.) μ-Porasil (Waters); stainless-steel (25 cm × 4.6 mm I.D.) packed with LiChrosorb RP-8 (Brownlee, Santa Clara, Calif., U.S.A.); stainless-steel (30 cm × 3.2 mm I.D. μBondapak C_{18} (Waters); stainless-steel (25 cm × 4.6 mm I.D.) Partisil-PXS-10/25ODS-2 (Whatman, Clifton, N.J., U.S.A.); stainless-steel (25 cm × 4.6 mm I.D.) Partisil-PXS-5/ODS (Whatman).

Solvents

Isooctane, chloroform and acetonitrile (Burdick & Jackson Labs., Muskegon, Mich., U.S.A.), diethyl ether (Mallinckrodt, St. Louis, Mo., U.S.A.) and methanol (Matheson, Coldeman & Bell, Norwood, Ohio, U.S.A.) were all residue-free and suitable for chromatography and spectrophotometry. Solvents were filtered through a 0.5-μm glassfiber filter (Gelman, Ann Arbor, Mich., U.S.A.) and degassed under vacuum prior to use.

Preparation of food samples

Extraction procedure. Six tomato samples (red-ripe Massachusetts greenhouse tomatoes), each weighing *ca.* 160 g, were individually cut into small pieces and homogenized under a stream of nitrogen in acetone for 1–2 min in a blender. The initial homogenate was filtered through a sintered glass funnel, pore size 20–30 μm (Whatman), under reduced pressure and the residue recovered for extraction. The procedure was repeated until complete extraction of all pigments was achieved. The acetone extract was then added to an equal volume of freshly distilled, peroxide-free, petroleum ether (PE) in a separatory funnel, mixed and diluted with water. Upon formation of two layers, the lower aqueous phase was re-extracted once with PE and the bulked PE solutions washed three times with water to remove acetone[3,4].

Saponification

Saponification is generally necessary in carotenoid analysis to remove unwanted lipid material which could interfere with the chromatography of compounds of interest.

Extracts were evaporated to dryness using a rotary evaporator, and a solution of 15% KOH in methanol was added to the round bottom flask. The alkaline mixtures were left in the dark for 14 h at room temperature. They were afterwards gradually added to freshly distilled petroleum ether in a separatory funnel. Water was slowly poured into the funnel so as not to form an emulsion. When two phases appeared, the lower aqueous phase was drawn off and extracted three times with fresh volumes of PE. The ethereal solutions were then bulked in a separatory funnel and washed free from alkali by repeated additions of water, followed by discarding the resultant aqueous layers. Each saponified extract was then concentrated to 100 ml in a rotary evaporator and stored under nitrogen in a volumetric flask[3,4].

Removal of sterols

The different samples were kept in the freezer overnight, at $-10°$, and the sterols precipitated to the bottom of the containers[3,4].

Aliquots for HPLC

Ten ml from each flask were filtered through a 0.5-μm glass-fiber filter before injection. Care was always taken not to expose any of the samples to light. All samples were stored under nitrogen in the freezer. Both 50-μl and 10-μl samples in petroleum ether were injected, the former to determine mainly β-carotene amounts and the latter, lycopene. Concentrations of each compound were determined from the slope of the calibration plots in which peak area was plotted against amount injected (nmoles β-carotene or lycopene). The detector responses were found to be linear over the entire working range.

Identification of peaks in HPLC eluents

Initial peak identification was based on retention times and comparison with the standards as well as co-chromatography with the standards. Since retention times alone are not sufficient for positive identification, stopped-flow visible spectra of the chromatographic peaks were also obtained. The Schoeffel variable wavelength detector is equipped with a memory module which automatically stores the spectral background

caused by changes in the optical properties of solvents, flow-cell light path and monochromator, and later subtracts it from the scans of the compounds. In order to obtain a stopped-flow scan, the flow is arrested at the top of each peak and the corrected spectrum scanned over the desired wavelength range.

RESULTS AND DISCUSSION

Optimization of chromatographic conditions

A Waters μPorasil (10-μm silica particles) was first tried. Pure isooctane, used as a mobile phase, resulted in long retention times (large k' values) and did not resolve α- and β-carotene (*cf.*, ref. 7); with addition of chloroform to isooctane both β-carotene and lycopene were eluted with the same retention time. Gradients were found to be impractical since the column re-equilibration necessitated more than 3 h under any set of conditions.

All other columns used had non-polar packings and were operated under reversed-phase conditions. The Brownlee column packed with LiChrosorb RP-8 (C_8 chemically bonded to silica, 10-μm particle size) resulted in too rapid elutions.

The increased "thickness" of the stationary phase in the C_{18}- over the C_8-coated packings is expected to increase the k' values[12]. This was indeed found to be true with Waters μBondapak C_{18} (10-μm particle size) and Whatman Partisil-PXS-10/25ODS-2 (also 10-μm particle size). The latter column and a mixture of 8.3% chloroform in acetonitrile as a mobile phase gave the best separation for a mixture of α- and β-carotene standards, although resolution ($R_s = 0.63$) was still inadequate.

TABLE I

INJECTIONS OF β-CAROTENE, α-CAROTENE AND LYCOPENE STANDARDS ON PARTISIL-10/ODS-2 AND PARTISIL-5/ODS

	Partisil-10/ODS-2		*Partisil-5/ODS*		
	Chloroform in acetonitrile		*Chloroform in acetonitrile*		
	11.6% *(1 ml/min)*	*8.5%* *(1 ml/min)*	*11.6%* *(2 ml/min)*	*8.5%* *(2 ml/min)*	*8.0%* *(2 ml/min)*
k'					
β-Carotene	6.0	8.48	5.84	7.90	8.84
α-Carotene	5.57	7.62	5.56	7.43	7.92
Lycopene	3.23	4.63	3.21	4.31	4.84
α					
$\frac{k'_{\beta\text{-Car}}}{k'_{\alpha\text{-Car}}}$	1.07	1.11	1.05	1.06	1.11
$\frac{k'_{\alpha\text{-Car}}}{k'_{\text{Lyc}}}$	1.72	1.64	1.73	1.72	1.63
$\frac{k'_{\beta\text{-Car}}}{k'_{\text{Lyc}}}$	1.85	1.83	1.82	1.83	1.82
$R_s = \frac{2(V_{\beta\text{-Car}} - V_{\alpha\text{-Car}})}{(W_{\beta\text{-Car}} + W_{\alpha\text{-Car}})}$		0.63	1.0	1.2	>1.5

Increasing the efficiency of the column by using 5-μm particles[13] in Whatman Partisil-PXS-5/ODS improved the resolution of the α- and β-carotene standards to a value of 1.2 (Table I). Samples were then chromatographed using this column and a mobile phase containing 8% chloroform in acetonitrile. Fig. 2 shows a chromatogram resulting from injecting the three standards simultaneously: the geometric isomers, α- and β-carotene, were resolved.

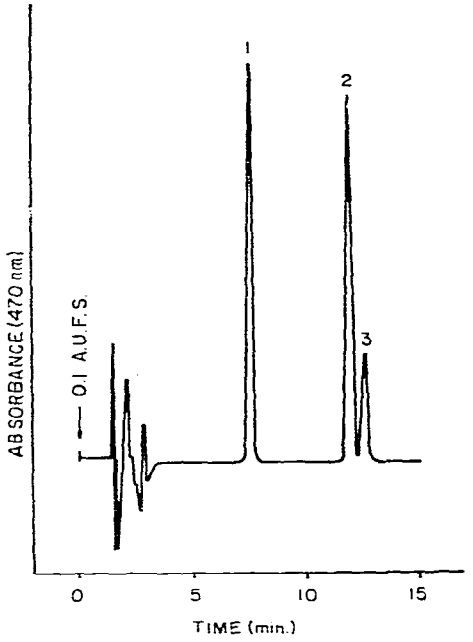

Fig. 2. Separation of standards: 1 = lycopene; 2 = α-carotene; 3 = β-carotene (R_s for peaks no. 2 and 3, 1.46). Chromatographic conditions: column, Partisil-5/ODS, 5 μm; eluent, 8.0% chloroform in acetonitrile; flow-rate, 2.0 ml/min; temperature, ambient; detection, 470 nm; sensitivity, 0.1 A.U.F.S.

Peak identification and quantitation

Under the chromatographic conditions used, the lower detection limits for lycopene, α-carotene and β-carotene standards were found to be 0.00395, 0.0372 and 0.0285 nmoles, respectively.

Chromatograms of tomato extracts run under the conditions described in the experiment are shown in Fig. 3. In reversed-phase chromatography, more polar compounds elute first[14]. This, together with classical literature on tomato pigments[15,17], indicates that the early eluted peak, X is probably a xanthophyll whereas peak Y, which elutes between lycopene and β-carotene, might be γ-carotene. α-Carotene is not prominent in any sample. As expected[15], the major components of tomato extracts, lycopene and β-carotene, were found to be in a *ca.* 9:1 ratio. Identification of these was done by comparing both the retention times and the visible spectra (380–600 nm) of the peaks from the extracts and the standard solutions. Close agreement between electronic absorption spectra (stopped-flow scanning method) confirms this identifi-

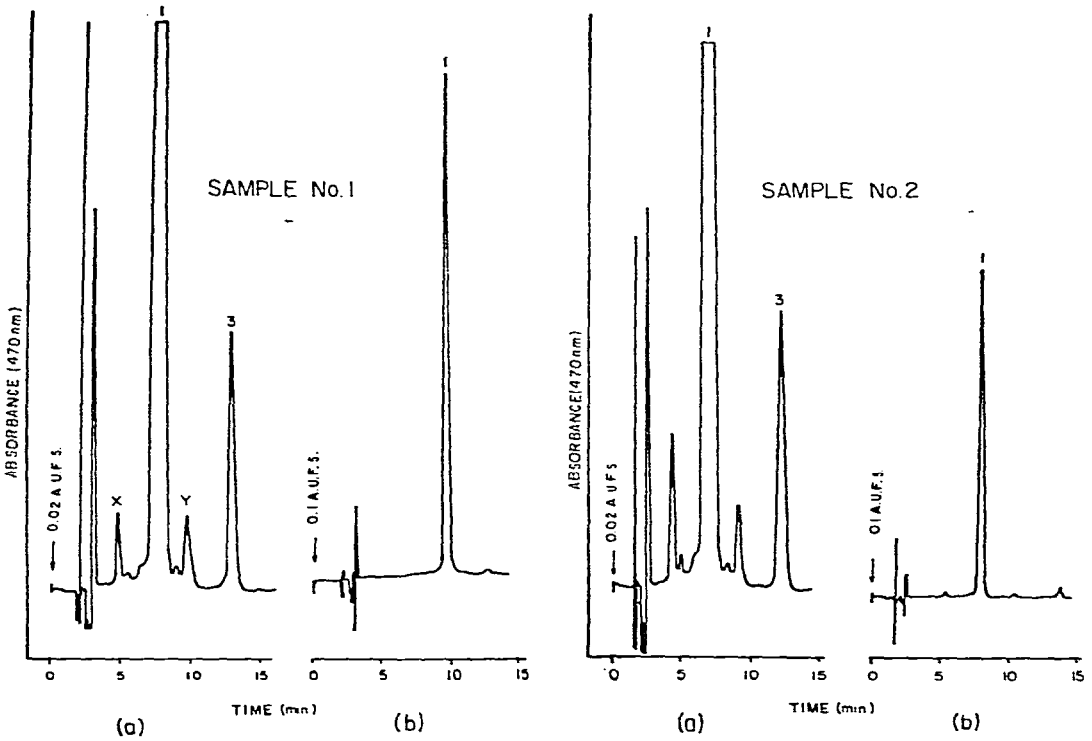

Fig. 3. Chromatograms of two tomato extracts: (a) 50 μl injected; (b) 10 μl injected. Chromatographic conditions as in Fig. 2. Peaks 1 and 3 are lycopene and β-carotene, respectively.

cation since the absorbance is a function of the chromophore[3], thus a characteristic of each carotenoid (Fig. 4).

The chromatographic peaks of β-carotene and lycopene were quantitated using the external calibration method; the values obtained for the tomato samples studied ware shown in Table II.

A report from the National Academy of Sciences estimates that only one sixth of the β-carotene from a diet is actually converted to retinol in humans[18]. The average β-carotene value of five samples, excluding sample No. 5, amounted to 1.218 μg/g or 1.218/6 = 0.2031 μg/g retinol equivalents. Since 0.3 μg retinol is by definition equivalent to 1 International Unit (I.U.), of vitamin A, the tomatoes studied contained 0.2031/0.3 = 0.6771 I.U./g.

The actual potency of the sample may be a little underestimated since γ-carotene, also a vitamin A precursor[16], was not accounted for. The resulting differences, however, should not be too important since γ-carotene is known to occur only in very small quantities in tomatoes[15,17] and has only one half the bioactivity of β-carotene[16].

On the other hand, including inactive pigments such as lycopene, which is present in large amounts in the tomato fruit, would overrate the provitamin A content of the sample. The method of the Association of Official Analytical Chemists (AOAC) which includes this carotenoid as well as others[8], results in erroneously high values (Table III).

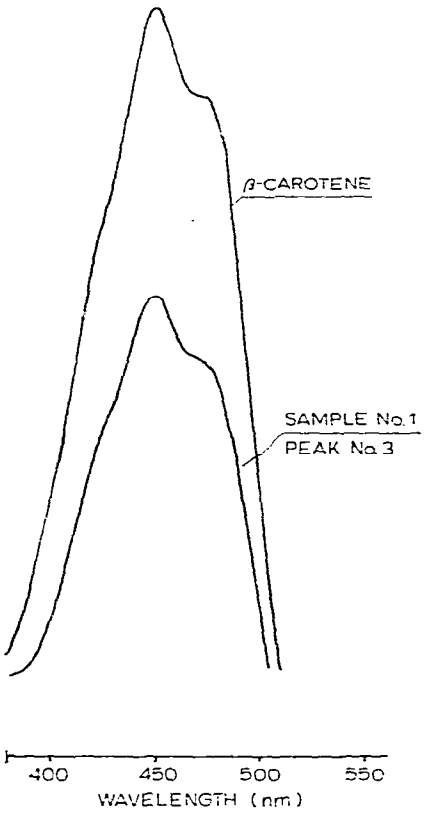

Fig. 4. Comparison of visible absorption spectra of β-carotene standard and peak no. 3 in sample no. 1. Scanning rate, 100 μm/min; sensitivity, 0.02 A.U.F.S.

TABLE II

QUANTITATION OF LYCOPENE AND β-CAROTENE IN TOMATO SAMPLES

Sample No.	*Lycopene (μg/g sample)*	*β-Carotene (μg/g sample)*
1	3.840	1.196
2	9.926	1.209
3	8.981	1.270
4	9.985	1.205
5	9.295	0.805
6	10.72	1.212

TABLE III

MEASUREMENT OF PROVITAMIN A VALUES IN TOMATOES: COMPARISON OF THE HPLC AND AOAC METHODS

In each case it is assumed that one sixth of the measured pigment(s) is converted to retinol.

Methodology	*Av. value (μg/g)*	*Retinol equivalent (μg/g)*	*I.U./g*
β-Carotene as obtained by HPLC	1.218	0.203	0.677
β-Carotene and lycopene obtained by HPLC	11.001	1.833	6.111
AOAC method	18.063	3.010	10.035

It is therefore of interest to quantitate vitamin A precursors only and to improve literature values which were obtained by using classical chromatographic and spectrophotometric techniques.

In summary, a new HPLC reversed-phase method was developed for the separation of carotenoids using a 5-μm particle column. The analysis of the saponified and washed extract is performed isocratically in less than 15 min. With the mobile phase of 8% chloroform in acetonitrile, the column pressure was found to be 2000 p.s.i.; it however, increased beyond usable range over period of time. The newly developed 5-μm packings, although more efficient than the regular 10-μm particles, have a shorter column life.

Repeated injections of standards and sample No. 1 were used to demonstrate the reproducibility of peak areas and retention times. The reversed-phase packings possess great stability and the ability to separate isocratically compounds of a wide polarity range (the acyclic lycopene being different from the bicyclic double-bond positional isomers α- and β-carotene).

In addition, nanogram quantities of compounds under study can be detected and since the analysis time is short the pigment decomposition and formation of artifacts are minimized[1]. All this makes the described HPLC system suitable for routine assays of provitamin A content in natural products. This is only a preliminary study and more work is necessary to insure the significance of quantitative data. This method can easily be applied to the analyses of various other vegetables and fruits, in order to determine the best natural sources of provitamin A, encourage their growth and promote their daily consumption.

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