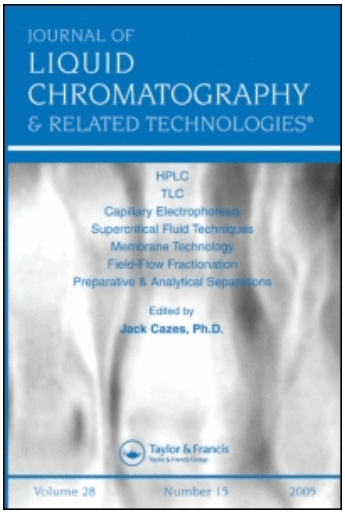

Journal of Liquid Chromatography & Related Technologies

Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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To cite this Article Bieri, John G. , Brown, Ellen D. and Smith Jr., J. Cecil(1985) 'Determination of Individual Carotenoids in Human Plasma by High Performance Liquid Chromatography', Journal of Liquid Chromatography & Related Technologies, 8: 3, 473 — 484
To link to this Article: DOI: 10.1080/01483918508067094
URL: http://dx.doi.org/10.1080/01483918508067094

JOURNAL OF LIQUID CHROMATOGRAPHY, 8(3), 473-484 (1985)

DETERMINATION OF INDIVIDUAL CAROTENOIDS IN HUMAN PLASMA BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

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ABSTRACT

A high performance liquid chromatography procedure using an internal carotenoid standard, echinenone, is described. The method uses a C-18, reverse-phase column, an isocratic solvent (acetonitrile:methylene chloride:methanol, 70:20:10) and requires only thirteen minutes. Most human plasmas gave seven distinct peaks; six carotenoids are identified. The sum of seven peaks is 90 percent or more of the total carotenoids in a total lipid extract.

 0148-3919/85/0803-0473 $3.50/0

INTRODUCTION

Recent speculation that the dietary intake of carotenoids may be a factor in the prevention of human cancer (2,3) has stimulated a renewed interest in carotenoid nutrition and metabolism. As part of a study to obtain more detailed information on carotenoid metabolism in normal humans, we have developed a rapid, quantitative analysis of plasma carotenoids based on the high performance liquid chromatographic (HPLC) system described by Nelis and De Leenheer (4). Previous HPLC methods for blood carotenoids (5,6,7,8, 9,10) have quantified one to three compounds (α- and β-carotenes and lycopene), whereas the method described here also determines lutein plus zeaxanthin (one peak), cryptoxanthin, and a previously undescribed plasma carotenoid.

MATERIALS AND METHODS

Instrumentation

The HPLC system was a Waters model M-6000A pump and model 440 absorbance detector with 436 nm filter, set at .01 AUFS, connected to either a Houston Instrument Omniscribe Recorder, or a Waters Data Module, model 730. The column contained a C-18, 5 micron reverse phase packing 25 cm x 4.6 mm (LC18, Supelco, Inc., Bellefonte, PA). The solvent was acetonitrile:

methylene chloride:methanol (70:20:10, v/v, ref. 4), run at 1.7 ml/min.

Standardization

Crystalline carotenoids used as standards (11) were: α- and β-carotenes, and lycopene (Sigma Chemical Co., St. Louis, MO); zeaxanthin, cryptoxanthin and echinenone (F. Hoffman-La Roche and Co., Basel, Switzerland). All compounds were estimated to be at least 95 percent pure from HPLC analysis. Stock solutions of carotenoids were prepared by dissolving 1-3 mg of the compounds in 2.0 ml of hexane, then diluting to 50 or 100 ml with absolute ethanol. If some of the carotenoids came out of solution they were filtered through a sintered funnel. These were diluted further to give working standards in the range of 0.4-1.0 μg/ml ethanol. These solutions were stable for at least three months at -20°C when protected from light. Concentrations were calculated from published absorptivity values ($E^{1\%}_{1\ cm}$) in hexane or ethanol (12,13) using a Cary model 219 spectrophotometer. Absorptivities in hexane generally vary from those in ethanol by only a few percent. Values used and the wavelength maxima, were: β-carotene, 2590 at 453 nm; α-carotene, 2800 at 440 nm; lycopene, 3450 at 472 nm; cryptoxanthin, 2370 at 452 nm; zeaxanthin, 2350 at 452 nm.

Standard curves plotting peak height ratios vs. weight ratios, using echinenone as an internal standard, were used to calculate response factors

$$RF = \frac{\text{conc of compound}}{\text{conc of standard}} \times \frac{\text{peak height of standard}}{\text{peak height of compound}}$$

Echinenone occurs in sea urchin eggs and algae (12,14) but is not usually found to any extent in the human food supply. We calculated an adsorptivity of 2160 at 458-462 nm in ethanol (15). The response factors for the various compounds under our conditions were: zeaxanthin, 0.78; unknown peak, 0.8 (assumed); pre-crytoxanthin, 0.83 (assumed); cryptoxanthin, 0.83; lycopene, 1.14; α-carotene, 1.37, and β-carotene, 1.64. The response factor for the unknown peak was assumed to be 0.8 because the peak is positioned between zeaxanthin and cryptoxanthin (the factors increase with greater elution times). Thus, a factor intermediate to zeaxanthin and cryptoxanthin seemed appropriate. We used the cryptoxanthin factor for the pre-cryptoxanthin peak which was on the front edge of cryptoxanthin.

Procedure

For the analysis of plasma, 100 µl plasma was pipetted into 6 x 50 mm test tubes followed by 100 µl echinenone internal standard solution in ethanol (ca.

0.2 μg/ml). The tube was vortexed for 20 sec., 120 μl hexane was added and the tube mixed vigorously for 40 sec. After centrifuging, 100 μl of the hexane phase was transferred to a second 6 x 50 mm test tube. This was placed in a 15-ml beaker of water at 60° and the hexane evaporated under N_2. The lipid was dissolved by mixing 30 sec. with 100 μl of the HPLC solvent, and 50-90 μl were injected depending on the amount of carotenoids present.

RESULTS AND DISCUSSION

A chromatogram of normal human plasma is shown in Fig. 1. The first peak is zeaxanthin, and is assumed to also contain the isomeric dihydroxy carotenoid, lutein. The second major peak has not been identified unequivocally (see below). The third major peak, cryptoxanthin, is preceded by a smaller peak, designated precryptoxanthin. The fourth major peak is lycopene. A space of one minute between cryptoxanthin and lycopene, with minimal interference, made it possible to use echinenone as an internal standard. The last two distinct peaks are α- and β-carotene, respectively. Minor peaks appear on the back of the lutein-zeaxanthin peak, and on the front of the unknown peak. These possibly may be cis isomers of the

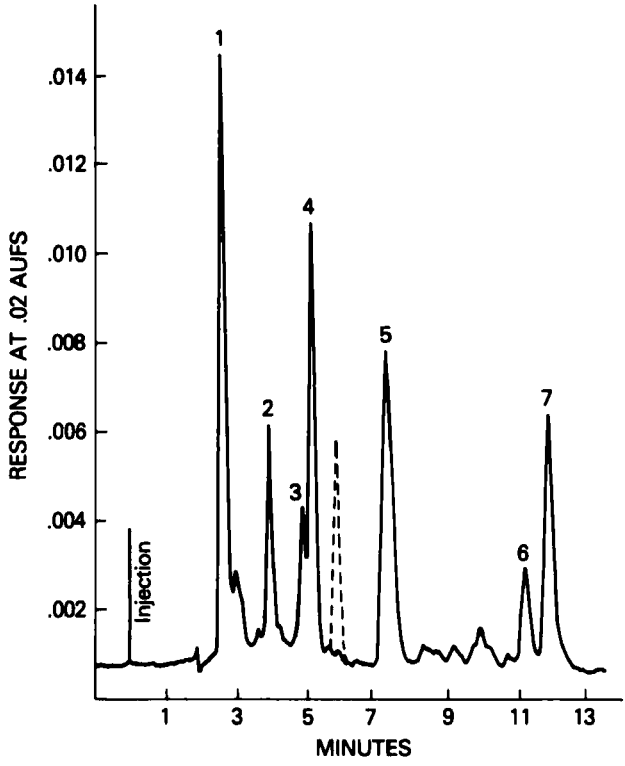

Figure 1. Representative HPLC chromatogram of normal adult plasma (solid line). Peaks are designated as follows: 1, lutein + zeaxanthin (L+Z); 2, unknown (X); 3, pre-cryptoxanthin (PC); 4, cryptoxanthin (C); 5, lycopene (Ly); 6, α-carotene (α-C); 7, β-carotene (β-C). The dotted line indicates the position of the internal standard, echinenone.

respective carotenoids. The small peaks between lycopene and α-carotene are also unidentified. The major unidentified peak (peak 2), has absorption maxima in ethanol of 443 nm and 470 nm, with a shoulder at 420 nm. These are close to the maxima for the

isomeric carotenoids violaxanthin and taraxanthin (zeaxanthin diepoxide and lutein diepoxide, respectively). We tentatively identify the unknown peak as either or both of these carotenoids.

The precision of the method for the same plasma analyzed eight times on the same day, or 8 times on consecutive days, is shown in Table 1. Recoveries of added carotenoids in this type of analysis can be misleading, since carotenoids added in a solvent are readily extractable in contrast to the lipoprotein-bound carotenoids in plasma. We felt that a better estimation of the adequacy of extraction would be given by comparing the sum of the peaks analyzed, to the total carotenoids in a lipid extract as determined classically. These data (Table 2) indicated that the seven peaks analyzed account for more than 90 percent of the total extractable carotenoids in plasma.

Results in a second laboratory using a Waters Data Module were comparable to those from the primary laboratory using an Omniscribe recorder and hand measurement for peak heights. This HPLC procedure for carotenoids now makes it possible to rapidly analyze the major carotenoids found in human plasma.

It should be recognized that the peaks may not be homogeneous for the predominantly all trans carotenoids

TABLE 1 Precision of the HPLC Procedure for Carotenoids

Peak	Same Day Analysis								Mean ± SD[2]	CV[3]
Lutein+Zeaxanthin	29.3	28.6	29.0	29.1	27.0	27.4	27.5	28.6	28.3±0.9	3.2
Unknown	14.8	14.1	13.1	12.6	13.0	13.1	12.2	13.3	13.3±0.8	6.0
Pre-cryptoxanthin	5.1	4.9	4.9	5.0	4.8	5.0	4.8	5.0	4.9±0.1	2.0
Cryptoxanthin	16.1	15.9	16.0	15.9	15.7	15.8	15.4	16.0	15.9±0.3	1.9
Lycopene	18.5	17.4	17.0	17.5	16.5	16.2	17.0	17.6	17.2±0.7	4.1
α-Carotene	6.4	6.3	5.9	5.8	5.5	5.5	6.0	6.0	5.9±0.3	5.1
β-Carotene	28.9	27.9	27.3	27.7	26.2	26.9	27.0	27.9	27.5±0.8	2.9
	Consecutive Days									
Day:	1	2	3	4	5	6	7	8	Mean ± SD[2]	CV
Lutein+Zeaxanthin	27.0	29.6	27.4	27.5	29.7	27.8	30.9	28.3	28.4±1.3	4.6
Unknown	12.6	14.1	13.1	12.8	13.5	12.2	14.6	13.5	13.3±0.8	6.0
Pre-cryptoxanthin	4.6	4.9	5.0	4.8	5.0	4.8	5.3	4.8	4.9±0.2	4.3
Cryptoxanthin	15.3	15.9	15.8	17.8	16.3	15.3	16.5	15.3	15.7±0.6	3.8
Lycopene	17.0	17.4	16.2	18.4	16.4	16.0	17.1	15.7	16.8±0.9	5.4
α-Carotene	5.8	6.3	5.5	5.6	6.0	5.6	6.5	5.7	5.9±0.4	6.8
β-Carotene	26.7	27.9	26.9	24.4	25.2	23.2	25.0	22.9	25.3±1.9	7.1

1. Values are averages of duplicate analyses.
2. Mean of eight analyses. Values are expressed as μg/dl plasma.
3. CV = coefficient of variation.

TABLE 2

Summation of Individual Plasma Carotenoids Compared to Total Carotenoids

Carotenoid Peaks[1]	Sample No. 1	2	3	4	5
Lutein + Zeaxanthin	18.1	32.4	41.2	17.1	31.5
Unknown	6.8	11.1	15.4	5.7	14.0
Pre-cryptoxanthin	4.6	5.7	10.0	3.2	3.8
Cryptoxanthin	7.4	17.3	30.3	9.4	12.5
Lycopene	20.9	28.6	28.5	61.9	20.0
α-Carotene	4.6	9.0	10.6	2.9	8.2
β-Carotene	13.4	33.5	32.5	16.8	45.6
Sum[2]	75.8	137.6	168.5	117.0	135.6
Total Carotenoids[3]	80.4	148.4	172.5	124.6	141.0
Sum as % of Total Carotenoid[4]	94.3	92.7	97.7	93.9	96.2

1. Values are μg/dl plasma from normal adults.
2. Summation of all peaks detected by present method.
3. Total carotenoids were determined as follows: 0.4 ml plasma mixed with 0.4 ml ethanol, then extracted with 0.8 ml heptane. After centrifugation, the absorbance of the heptane extract was read at 445 nm. Concentration calculated for A_{453} using absorptivity of 2590 (β-carotene).
4. $\frac{\text{Sum}}{\text{Total Carotenoids}} \times 100$

found in plants, but that cis-isomers also may be present. For example, using a Hewlett Packard 1040A photodiode array detector it was shown that 15, 15'-cis-β-carotene appeared at the tail end of the β-carotene peak. This mono-cis-isomer was identified

by its distinct absorption spectrum which had a strong cis peak at 340 nm and a maximum at 447 nm.

The method is intended as a screening technique for nutritional or clinical studies where the pro-vitamin A status is of interest. The possible presence in human plasma of a significant amount of an epoxy carotenoid is a new and interesting observation. Further study will be needed to precisely characterize this peak and to ascertain its dietary origin.

ACKNOWLEDGEMENTS

The authors wish to thank F. Hoffmann-La Roche, Basle, Switzerland for samples of carotenoids., Dr. Van Hubbard, National Institue of Arthritis, Diabetes, Digestive and Kidney Diseases, for plasma samples, and Dr. Fred Khachik U.S.D.A. for identification of the cis-isomer of β-carotene. This work was funded in part by the National Cancer Institute, Interagency Cooperative Agreement Y01-CN-4-0620.

REFERENCES

1. Author to whom reprint requests should be sent: J. Cecil Smith, Jr., U.S. Department of Agriculture, ARS, Beltsville Human Nutrition Research Center, Vitamin and Mineral Nutrition Laboratory, Beltsville, Maryland 20705.

2. Peto, R., Doll, R., Buckley, J.D. and Sporn, M.B. Can Dietary β-Carotene Materially Reduce Human Cancer Rates? Nature 290, 201, 1981.

3. Shekelle, R.B., Liv, S., Raynor, W.J., Lepper, M., Maliza, C., Rossof, A.H., Paul, O., Shryock, A.M., and Stamler, J. Dietary Vitamin A and the Risk of Cancer in the Western Electric Study. Lancet ii, 1185, 1981.

4. Nelis, H.J.C.F. and De Leenheer, A.P. Isocratic, Nonaqueous Reversed-phase Liquid Chromatography of Carotenoids. Anal. Chem. 55, 270, 1983.

5. Broich, C.R., Gerber, L.E. and Erdman, J.W., Jr. Determination of Lycopene, α- and β-Carotene and Retinyl Esters in Human Serum by Reversed-Phase High Performance Liquid Chromatography. Lipids 18, 253, 1983.

6. Driskell, W.J., Bashor, M.M. and Neese, J.W. Beta-carotene Determined in Serum by Liquid Chromatography with an Internal Standard. Clin. Chem. 29, 1042, 1983.

7. Peng, Y. and Beaudry, J. High-Performance Liquid Chromatography of the Provitamin A β-Carotene in Plasma. J. Chromatogr. 273, 410, 1983.

8. Vuilleumier, J.P., Keller, H.E., Gysel, D. and Hunziker, F. Clinical Chemical Methods for the Routine Assessment of the Vitamin Status in Human Population. Internat. J. Vit. Nutr. Res. 53, 265, 1983

9. Katrangi, N., Kaplan, L.A. and Stein, E.A. Separation and Quantitation of Serum β-Carotene and Other Carotenoids by High Performance Liquid Chromatography. J. Lipid Res. 25, 400, 1984.

10. Miller, K.W., Lorr, N.A., and Yang, C.S. Simultaneous Determination of Plasma Retinol, α-Tocopherol, Lycopene, α-Carotene and β-Carotene by High-Performance Liquid Chromatography. Anal. Biochem. 138, 340, 1984.

11. Trivial names and IUPAC-IUB nomenclature, respectively, are: lutein, 3,3'-dihydroxy-β, ε-carotene; zeaxanthin, 3,3'-dihydroxy-β-β-carotene;

cryptoxanthin, 3-hydroxy-β,β-carotene; echinenone, 4-keto-β, β-carotene; lycopene, ψ,ψ -carotene; α-carotene, β, ε -carotene; β-carotene, β,β-carotene.

12. Goodwin, T.W. The Comparative Biochemistry of the Carotenoids. Chapman and Hall, Ltd, London. 1952.

13. Sebrell, W.H., Jr. and Harris, R.S. The Vitamins. Vol. I, 2nd Ed. Academic Press, New York, 1967.

14. Akhtar, M. and Weedon, B.C.L. Carotenoids and Related Compounds. Part VIII. Novel Syntheses of Echinenone and Canthoxanthin. J. Chem. Soc. 4058, 1959.

15. Ganguly, J., Krinsky, N.I. and Pinckard, J.H. Isolation and Nature of Echinenone, a Provitamin A. Arch. Biochem. Biophys. 60, 345, 1956.