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Determination of Retinol, α-Tocopherol, α- and β-Carotene by Direct Extraction of Human Serum Using High Performance Liquid Chromatography

Monira A. Abahusain*[1], John Wright[2], John W. T. Dickerson[3], Mohesen A. El-Hazmi[3] and Hassan Y. Aboul Enein[4]

[1] Department of Family and Community Medicine, College of Medicine, King Saud University, PO Box 2925, Riyadh 11461, Saudi Arabia
[2] School of Biological Sciences, University of Surrey, Guildford, Surrey GU2 5XH, UK
[3] Medical Biochemistry Department, College of Medicine, King Saud University, PO Box 2925, Riyadh 11461, Saudi Arabia
[4] Bioanalytical and Drug Development Laboratory, Biological and Medical Research Department, MBC-03, King Faisal Specialist Hospital and Research Center, PO Box 3354, Riyadh 11211, Saudi Arabia

In this report we describe a modified reverse phase HPLC method that avoids the solvent evaporation step and allows simple and rapid determination of retinol, α-tocopherol, α-carotene and β-carotene and achieves complete separation of α- and β-carotene. Retinyl acetate, α-tocopheryl acetate and retinyl palmitate in ethanol were added to serum as internal standards. Serum was then deproteinized with an equal volume of ethanol, and the lipid was extracted with ethyl acetate–butanol (1:1 v/v). A portion of this solution was injected into a C18 reverse phase chromatographic column and absorbencies of the vitamins and internal standards were measured at 292 nm for tocopherols, 325 nm for retinoids and 450 nm for carotenoids; peak–height ratios were used to quantify each vitamin. The analytical recoveries for retinol, α-tocopherol α- and β-carotene at various concentrations tested were 95–103, 90–98, 92–99 and 94–96%, respectively. The intra- and interassay variations for low and high concentrations of retinol, α-tocopherol, α- and β-carotene ranged from 2.4 to 6.7 for intraassay and from 4.3 to 8.5 for interassay replication. The detection limits were 1.25 (0.04), 19 (0.44), 0.35 (0.006) and 0.94 (0.017) μg/dL (δmol/L) for retinol, α-tocopherol, α- and β-carotene, respectively.

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Keywords: HPLC; human serum; α-tocopheral; retinol; α- and β-carotene

INTRODUCTION

Clinical interest in evaluation of retinol, α-tocopherol, and β-carotene has increased in recent years owing to the possible roles of these nutrients as antioxidants which may be important in reducing risk of a number of diseases including cancer, coronary heart disease and cataract (Esterbauer *et al.*, 1989; Riemersma *et al.*, 1991; Canfield *et al.*, 1992; Harris *et al.*, 1992; Gottlieb *et al.*, 1993; Hennekens and Gaziano, 1993). Thus, a rapid, simple and specific method for determination of these compounds in serum or plasma is desirable.

High performance liquid chromatography (HPLC) methods are considered superior for the determination of these nutrients. Many of these methods are time-consuming and require extraction of the analytes from serum or plasma into an organic solvent, which is then evaporated to dryness (De Leenheer *et al.*, 1979; Catignani and Bieri, 1983; Driskell *et al.*, 1983; Miller *et al.*, 1984; Bieri *et al.*, 1985; Milne and Botenen, 1986; Kaplan *et al.*, 1990; Nierenberg and Nann, 1992; Zaman *et al.*, 1993; Bui, 1994; Lanvers *et al.*, 1996).

Extraction methods that do not need this evaporation step would offer an advantage. Such methods have been reported (De Ruyter and De Leenheer, 1976; Nierenberg, 1984; Kalman *et al.*, 1987; Lee *et al.*, 1992; Siddiqui *et al.*, 1995) but some of these reports described attempts to determine retinol only (De Ruyter and De Leenheer, 1976; Nierenberg, 1984; Siddiqui *et al.*, 1995), while in another study, (Lee *et al.*, 1992), the peaks of α- and β-carotene were not totally separated. We therefore attempted the development of a simple and rapid extraction procedure, incorporating extraction with butanol–ethyl acetate, that allows direct injection into the HPLC system, together with a different mobile phase that achieves a simultaneous determination and complete separation of retinol, α-tocopherol, α- and β-carotene.

EXPERIMENTAL

Reagents and chemicals. Trans retinol, retinyl acetate, α-tocopherol, α-tocopheryl acetate, α-carotene, β-carotene and retinyl palmitate were all obtained from Sigma Chemical Company (Poole, Dorset, England). Sodium sulphate anhydrous was obtained from BDH (Poole, England).

All solvents used were HPLC grade or better. Acetonitrile, ethylene chloride, methanol and ethyl acetate were obtained from Fisher Chemical Company (Fair Lawn, New Jersey, USA).

* Author to whom correspondence should be addressed.

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Butanol and ethanol were obtained from Aldrich (Milwaukee, USA) and BDH (Poole, England) respectively.

Instrumentation and chromatographic condition. The HPLC system was from Waters Associates (Milford, Mass, USA) and consisted of an automated sample injector (WISP 712B), a Model 510 pump, a Model 490 multi-wavelength detector, a Model 745 data integrator modules, a Gradient controller 860 and a Guard-pak precolumn cartridge with disposable inserts of 5 μm.

Supelco stainless steel column (25 cm × 4.6 mm, i.d.) and precolumn (5 cm × 4.6 mm, i.d.) packed with ODS Supelcosil LC 18, 5 μm particles, were obtained from Supelco (Supelco–Sigma–Aldrich; Poole, England)). Dual pen recorder was obtained from LKB (LKB, Bromma, Sweden).

The mobile phase used was a mixture of acetonitrile–methylene chloride–methanol (75:15:10 by vol). It was degassed under vacuum prior to use and pumped at a flow rate of 1.0 mL/min for retinoids and tocopherols and 1.4 mL/min for carotenoids. With the help of a gradient controller, the flow rate was increased from 1 mL/min for 14 min to 1.4 mL/min until the end of the run. The retinoids, tocopherols and cartenoids were monitored at 325, 292 and 450 nm, respectively. The absorption unit full scales (Aufs) were 0.025 for the retinoids and tocopherols and 0.005 for the carotenoids.

Standards and standard curve preparation. Standard solutions of the following analytes were prepared by dissolving, in ethanol, trans retinol and retinyl acetate (each to 1 mg/mL), α-tocopheryl acetate (each to 25–40 mg/mL), and in chloroform, α- and β-carotene (each to 0.5 mg/mL) and retinyl palmitate (30–40 mg/mL). The concentrations, in ethanol, were determined spectrophotometrically using Beer's Law. The absorptivity coefficient values (E1%/cm) used were 1780 at 325 nm for retinol, 75.8 at 292 nm for α-tocopherol, 1510 at 325 nm for retinyl acetate, 43.4 at 285 nm for α-tocopheryl acetate, 2800 and 2620 for α- and β-carotene, respectively at 450 nm. The standards were prepared under red light conditions and stored at −20°C when not in use. The stock solutions were prepared every month, and fresh standards were prepared from the stock solutions for each assay. To prepare a standard curve, a constant amount of internal standard and 4 different concentrations of the analytes, in ethanol, were added to serum. Five duplicates of serum standards were prepared by adding 10 microliters of combined working standards in the range of 4–32, 80–640, 2–16, and 0.4–3.2 μg/mL for retinol, α-tocopherol, β-carotene and α-carotene, respectively, and 10 microliters of combined internal standard of 2.4 mg/mL for α-tocopheryl acetate, 30 μg/mL for retinyl acetate and 40 μg/mL of retinyl palmitate to 200 microliters serum. No solutions were added to the fifth set of tubes.

Sample collection and preparation. A single fasting venous blood sample was obtained from all subjects. The blood was drawn into 10 ml vacutainer tubes and samples were kept in the dark before centrifugation at 1500 *g* for 10 min at 4°C. Serum was removed and each sample was divided into 500 μL aliquots and stored at −70°C until analysis.

The samples were prepared by addition of 10 μL mixed internal standard to each tube containing 200 μL of serum. The mixture was vortexed for 10 seconds and allowed to stand in a cool dark place for 20–30 minutes for equilibration to occur. Absolute ethanol was added to each tube to make a final volume of 400 μL.

Extraction. Four hundred microliters of ethyl acetate–butanol (1:1 v/v) were added. The solution was mixed using a vortex mixer for 1 minute. Approximately 20 mg of sodium sulphate were added.

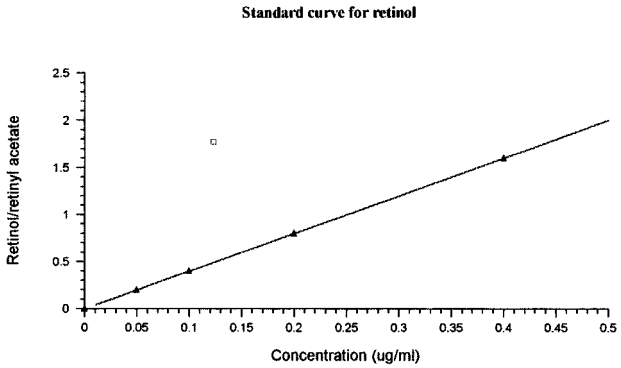

Figure 1. Calibration curve for retinol concentration versus peak heights ratio. Known amounts of standard were added to human serum. Each point represents the mean of duplicate samples. $y = 3.51\ x + 0.0045$; $r = 1.00$.

The mixture was vortex-mixed for a further minute and allowed to stand at −20°C for 20 minutes before centrifugation at 1500 *g* for 10 minutes at 4°C. The organic phase was transferred into injection vials and 40 microliters were injected into the HPLC. All samples were treated in duplicate, and analyte concentrations were determined from a standard curve of the peak height ratio of the analyte/internal standard plotted against the concentration of analyte. Calibration graphs and analysis of linearity were performed by linear least-square regression analysis. Typical standard curves are shown in Figs 1 and 2. Standard curves were constructed after correction for the contribution of endogenous analyte in the serum. Regression analysis was performed for each analyte and if the regression coefficient was below 0.99 the standard curve was repeated.

Quality control. Pooled normal human serum samples of endogenous concentration of 23.4 μg/dL (0.82 μmol/L) for retinol, 5.2 μg/mL (12 μmol/L) for α-tocopherol, 3.75 μg/dL (0.07 μmol/L) for α-carotene and 15.0 μg/dL (0.28 μmol/L) for β-carotene was divided into two portions. One portion was used as the low concentrations of analytes. The other portion was spiked with 25 μg/dL (0.87 μmol/L) retinol, 6.0 μg/mL (13.9 μmol/L) α-tocopherol, 4.0 μg/dL (0.074 μmol/L) α-carotene, 20 μg/dL (0.37 μmol/L) β-carotene to produce known high concentrations of analytes. These portions were mixed thoroughly then aliquots of 450 microliters were dispensed into 1.5 mL microcentrifuge tubes and stored at −70°C. These quality control (QC) samples were used for each assay. They were prepared for analysis by the same procedure for the unknown and standard samples. Evaluation of laboratory performance was assessed by comparing results of the

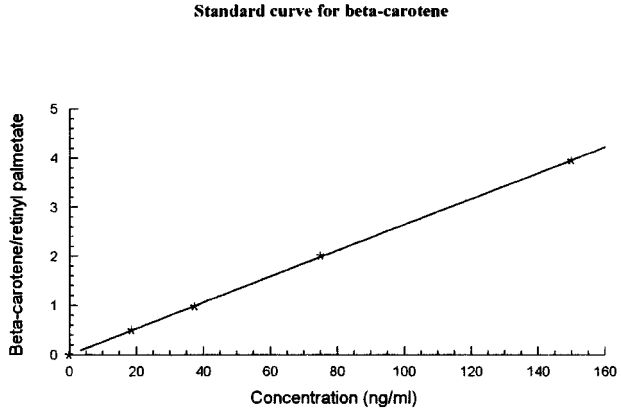

Figure 2. Calibration curve for beta-carotene concentration versus heights ratio. Known amounts of standard were added to human serum. Each point represents the mean of duplicate samples. $y = 0.0264\ x + 0.0045$; $r = 0.9999$.

Table 1. Variations in estimation of retinol, α-tocopherol, α- and β-carotene

	INTRAASSAY (N=10)							
Concentration	Retinol (γg/dL) Mean (±SD)	CV%	Alpha-tocopherol (μg/dL) Mean (±SD)	Beta-carotene (μg/dL) CV%	Alpha-carotene (μg/dL) Mean (±SD)	CV%	Mean (±SD)	CV%
Added	0		0		0		0	
Found	23.4 (0.7)	3.0	52 (1.25)	2.4	15 (1.0)	6.7	3.75 (0.135)	4.4
Added	25		60		20		4	
Found	47.3 (2.17)	4.6	110 (3.2)	2.9	34.8 (2.1)	6.0	7.69 (0.47)	6.2
	INTERASSAY (N=25)							
Added	0		0		0		0	
Found	22.8 (1.1)	4.8	52 (2.2)	4.3	16.85 (1.18)	7.0	3.75 (0.28)	5.7
Added	25		60		20		4	
Found	46.9 (2.9)	6.2	112 (5.8)	5.2	37.7 (3.2)	8.5	7.74 (0.51)	6.7

quality control (QC) samples with the mean and standard deviations that were calculated using results from several preliminary runs. If any value occurred outside the range (±2 standard deviations) from the mean, the run was rejected.

RESULTS AND DISCUSSION

It was thought desirable to develop an extraction process that avoids a solvent evaporation step. This extraction procedure was a modification of the method established by De Ruyter and De Leenheer (1976). It allows direct injection into the HPLC system. De Ruyter and De Leenheer attempted to determine retinol only (1976). Lee *et al.*, (1992) achieved simultaneous separation of retinol, α-tocopherol, α- and β-carotene. However, base line resolution of α- and β-carotene was not achieved (Lee *et al.*, 1992). Extracted analytes were found to be stable in the extraction solvent (ethyl acetate–butanol) after dehydration with sodium sulphate (Lee *et al.*, 1992). The stability of analytes allows a large number of samples to be extracted and stored for later HPLC analysis. Plasma or serum samples with these nutrients can be stored up to two years at −20°C for retinol and α-tocopherol and for five months at −20°C for α- and β-carotene without loss of stability (Craft *et al.*, 1988). This method has sufficient sensitivity and precision; detection limits were 1.25 (0.04), 19 (0.44), 0.35 (0.006) and 0.94 (0.017) μg/dL (μmol/L) for retinol, α-tocopherol α- and β-carotene respectively. Detection limit was determined by analysing a sample with analyte concentration three to five times the noise level, ten consecutive times. Sensitivity could be increased by increasing the sensitivity of the UV detector, or by increasing the volume injected. However, there was no need for either measure as the normal concentrations of the analytes are well above the detection limits. These detection limits demonstrate good sensitivity for the method.

Table 1 summarizes intra- and interassay variations for low and high concentrations of retinol, α-tocopherol, α- and β-carotene. The coefficient of variation (CV) of the determinations ranged from 2.4 to 6.7 for intraassay and 4.3 to 8.5 for interassay replication. These were comparable with those reported by others (Catignani and Bieri, 1983; Bieri *et al.*, 1985; Milne and Botnen, 1986; Lee *et al.*, 1992;

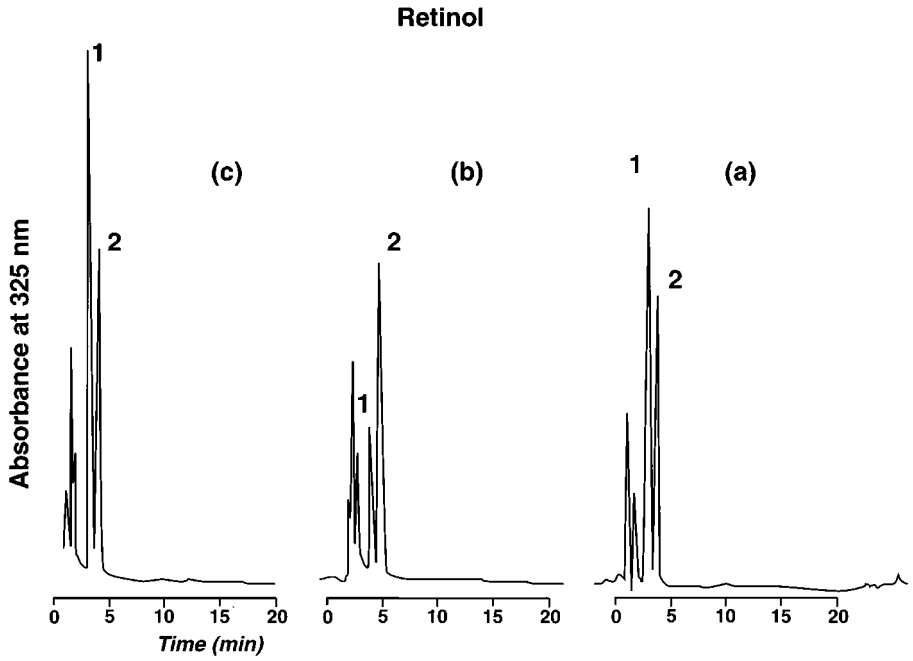

Figure 3. Chromatogram of (a) pure standard of retinol and its internal standard retinyl acetate, (b) blank serum with internal standard, (c) the same serum spiked with concentration as in (a), peaks: 1=retinol; 2=retinyl acetate. Separation was obtained by Supelcosil packed with 5 μm LC 18 (25 cm × 4.6 mm, i.d.) column. Eluent: acetonitrile–methylene chloride–methanol (75:15:10, v/v/v). The flow rate was 1.0 mL/min for 14 min, then 1.4 mL/min for the next 10 min.

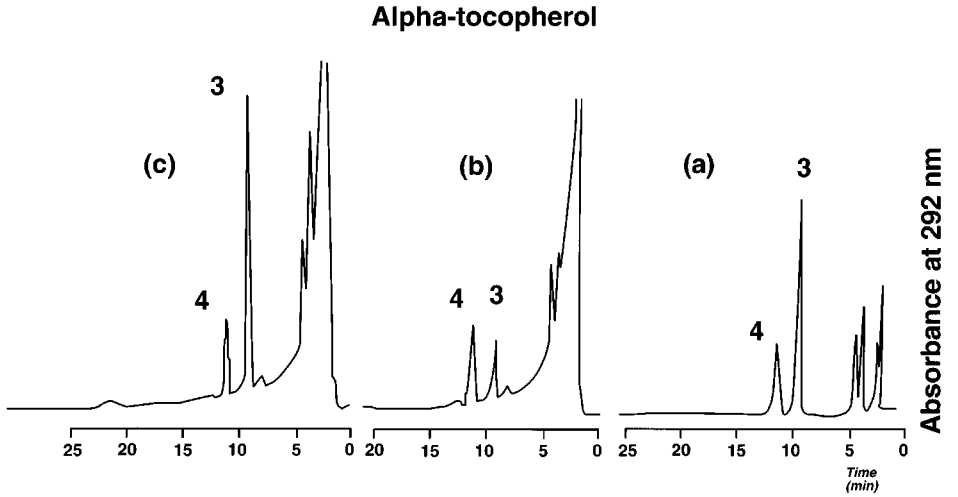

Figure 4. Chromatogram of (a) pure standard of α-tocopherol and its internal standard α-tocopheryl acetate, (b) blank serum with internal standard, (c) the same serum spiked with concentration as in (a), peaks: 3 = α-tocopherol; 4 = α-tocopheryl acetate. Separation was obtained by Supercosil packed with 5 μm LC 18 (25 cm × 4.6 mm, i.d.) column. Eluent: acetonitrile–methylene chloride–methanol (75:15:10, v/v/v). The flow rate was 1.0 mL/min for 14 min, then 1.4 mL/min for the next 10 min.

Nierenberg and Nann, 1992; Zaman *et al.*, 1993). The CV was, in all cases, less than 10%.

Analytical recovery was measured by adding known amounts of analytes into 36 extraction tubes (A, B and C). These extraction tubes were taken through the method. The recovery of the added anlytes was expressed as a percentage. The analytical recovery for retinol, α-tocopherol and α- and β-carotene at various concentrations tested was 95–103, 90–98, 92–99 and 94–96%, respectively, being lower in the low concentration range (Table 2). Recovery was consistent and satisfactory and was comparable with reported values (De Leenheer *et al.*, 1979; Driskell *et al.*, 1982; Milne and Botnen, 1986; Lee *et al.*, 1992).

This method has clear advantages over several methods

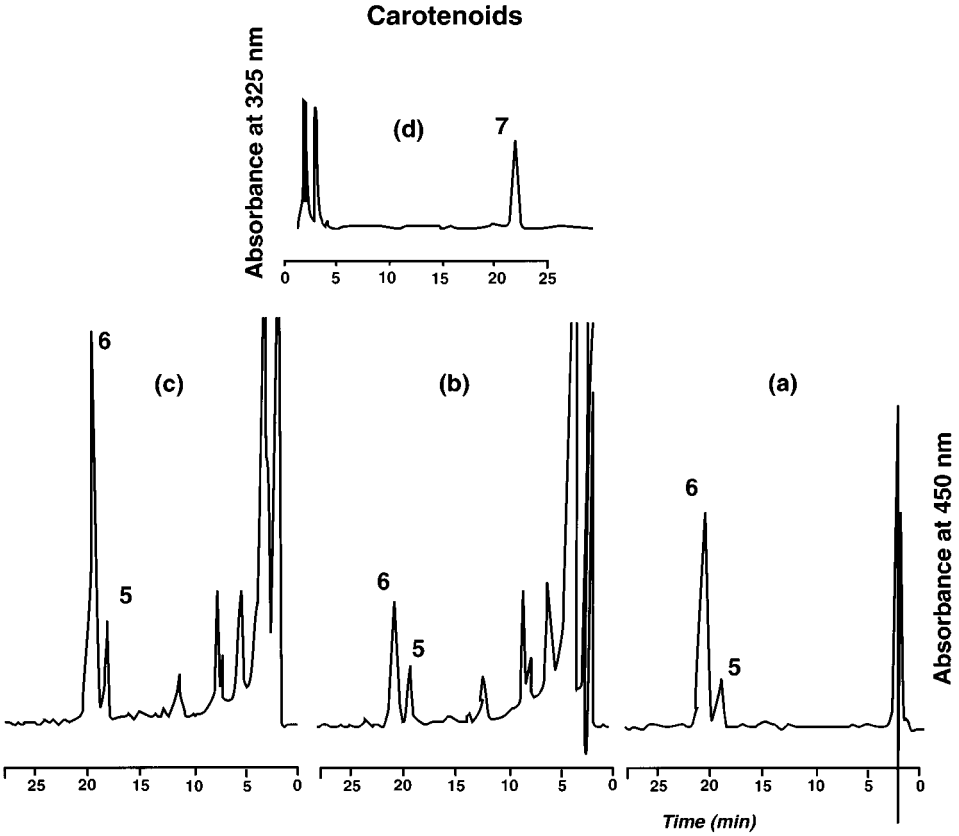

Figure 5. Chromatogram of (a) pure standard of α- and β-carotene and its internal standard retinyl palmitate, (b) blank serum with internal standard, (c) the same serum spiked with concentration as in (a), peaks: 5 = β-carotene; 6 = α-carotene; 7 = retinyl palmitate. Separation was obtained by Supercosil packed with 5 μm LC 18 (25 cm × 4.6 mm, i.d.) column. Eluent: acetonitrile–methylene chloride–methanol (75:15:10, v/v/v). The flow rate was 1.0 mL/min for 14 min, then 1.4 mL/min for the next 10 min.

Table 2. Recovery of retinol, α-tocopherol, α- and β-carotene from pooled serum by ethyl acetate–butanol (1:1) extraction

Concentration	Retinol (μg/dL)	Alpha-tocopherol (μg/dL)	Alpha-carotene (μg/dL)	Beta-carotene (μg/dL)
Initial	21.3 (0.42)	62.5 (1.9)	0.68	5.9 (0.03)
Added (A) $n=12$	20.0	60	1.0	10.0
Found (Mean ± SD)	39.2 (1.57)	110 (3.5)	1.55 (0.05)	14.9 (0.49)
Recovery %	95	90	92	94
Added (B) $n=12$	60.0	200	4.0	20.0
Found (Mean ± SD)	83.7 (5.3)	257.2 (20.2)	4.63 (0.18)	24.86 (0.63)
Recovery %	103	98	99	96
Added (C) $n=12$	120	400	8.0	40.0
Found (Mean ± SD)	142.3 (5.3)	444 (23)	8.16 (0.65)	43.6 (1.8)
Recovery %	101	96	94	95

in which satisfactory recovery was achieved only after two extractions (MacCrehan and Schonberger, 1987; Zaman *et al.*, 1993). Elution profiles of the fat soluble vitamins extracted from serum with internal standards are shown in Figs. 3(b), 4(b), 5(b). They compare well with chromatograms of pure standards [Figs 3(a), 4(a), 5(a)]. The figs 3(c), 4(c), 5(c) also show the chromatograms obtained from the same serum spiked with known concentrations of standards. Comparison of the respective retention times and absorption spectra with those of pure standards established the peak identities. Retinol, retinyl acetate and retinyl palmitate monitored at 325 nm eluted at 4.3, 4.9 and 22.7 min, respectively. Alpha-tocopherol and α-tocopheryl acetate monitored at 292 nm eluted at 10.0 and 11.5 min, respectively, α- and β-carotene monitored at 450 nm eluted at 19.5, and 20.8 min, respectively. The relative selectivity $\alpha = (k'2/k'I))$ for retinol and retinyl acetate = 1.5, and for α-tocopherol and α-tocopheryl acetate = 1.2. The relative selectivity for α- and β-carotene is 1.1 indicating good resolution.

CONCLUSION

In conclusion, the method described gives excellent recoveries and improved resolution of α- and β-carotene. The sample preparation and analytical time is relatively short. The method is suitable for routine analysis and can be performed automatically using a multiwave length detector. It can be of use in clinical or epidemiological studies where these vitamins are of interest.

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REFERENCES

Bieri, J. G., Brown, E. D. and Smith, J. C. Jr. (1985). *J. L. Chromatogr.* **8**(3), 473.
Bui, M. H. (1994). *J. Chromatogr. B.* **654**, 129.
Canfield, L. M., Forage, J. W. and Valenzuela, J. G. (1992). *Proc. Soc. Exp. Biol. Exp.* **200**, 260.
Catignani, G. L. and Bieri, J. G. (1983). *Clin. Chem.* **29**(4), 708.
Craft, N. E. Brown, E. D. and Smith, J. C. Jr. (1988). *Clin. Chem.* **34**(1), 44.
De Leenheer, A. P., Bevere, V. O., De Ruyter, M. G. and Claeys, A. E. (1979). *J. Chromatogr.* **162**, 408.
De Ruyter, M. G. M. and De Leenheer, A. P. (1976). *Clin. Chem.* **22**(10), 1593.
Driskell, W. J., Bashor, M. M. and Neese, J. W. (1983). *Clin. Chem.* **29**(6), 1042.
Esterbauer, H., Striegl, G., Puhl, H., Oberreither, S., Rotheneder, M., El-Saadani, M. and Jurgens, G. (1989). *Ann. N Y Acad. Sci.* **570**, 254.
Gottlieb, K., Zarling, E. J., Mobarhan, S., Bowen, P. and Sugurman, S. (1993). *Nutr. Cancer* **19**, 207.
Harris, W. S. (1992). *Clin. Cardiol.* **15**, 636.
Hennekens, C. H. and Gaziano, M. (1993). *Clin. Cardiol.* **16** (Suppl. I), 110.
Kalman, D. A., Goodman, G. E., Omenn, G. S., Bellamy, G. and Rollins, B. (1987). *J. Nat. Cancer Inst.* **79**(5), 975.
Kaplan, L. A., Miller, J. A., Stein, E. A. and Stampfer, M. J. (1990). *Methods in Enzymology.* **189**, 155.
Lanvers, C., Hempel, G., Blaschke, G. and Boos, J. (1996). *J. Chromatogr. B.* **685**, 233.
Lee, B. L., Chua, S. C., Ong, H. Y. and Ong, C. N. (1992). *J. Chromatogr.* **581**, 41.
MacCrehan, W. A. and Schonberger, E. (1987). *Clin. Chem.* **33**(9), 1585.
Miller, K. W., Lorr, N. A. and Yang, C. S. (1984). *Anal. Biochem.* **138**, 340.
Milne, D. B. and Botnen, J. (1986). *Clin. Chem.* **32**(5), 874.
Nierenberg, D. W. and Nann, S. L. (1992). *Am. J. Clin. Nutr.* **56**, 417.
Nierenberg, D. W. J. (1984). *Chromatogr.* **311**, 239.
Riemersma, R. A., Wood, D A., Macintyre, C. C. A., Elton, R. A., Gey, K. F. and Oliver, M. F. (1991). *The Lancet* **337**, 1.
Siddiqui, F. Q., Malik, F. and Fazli, F. R. Y. (1995). *J. Chromatogr. B.* **666**, 342.
Zaman, Z., Fielden, P. and Frost, P. G. (1993). *Clin. Chem.* **39**(10), 2229.

