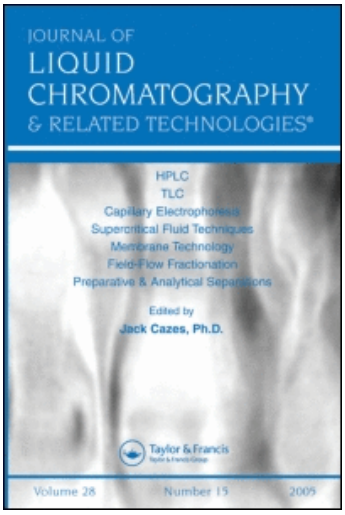

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Adaptation of a Carotenoid Procedure to Analyze Carotenoids, Retinol, and Alpha-Tocopherol Simultaneously

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ADAPTATION OF A CAROTENOID PROCEDURE TO ANALYZE CAROTENOIDS, RETINOL, AND ALPHA-TOCOPHEROL SIMULTANEOUSLY

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ABSTRACT

An isocratic procedure for carotenoids (Nelis and De Leenheer, 1983) was extended by monitoring at an extra wavelength (280 nm) and use of tocopheryl acetate as internal standard to include retinol (vitamin A) and α-tocopherol (vitamin E) with carotenoid analysis in a single chromatographic run. Peaks were validated by retention times, spiked test and signal ratio (313 nm/280 nm). Within-run CVs (n=10) were 4.7% and 2.9% and between-run CVs (n=9) were 7.3% and 5.1% for vitamins A and E respectively. Linearities were up to at least 1.8 ug/mL (vitamin A) and 46.5 ug/mL (vitamin E). Vitamins A and E of twenty serum samples were analyzed by this procedure (I) and a reference procedure (II) and their correlations were 0.8484 and 0.9919 respectively. Linear regressions were I=II(0.806)+0.225 ug/mL (vitamin A) and I=II(0.974)+0.52 ug/mL (vitamin E). Recoveries were 118% and 111% for vitamins A and E respectively. This isocratic procedure can analyze at least five carotenoids and vitamins A and E in serum simultaneously. It is simple and economic in time and reagents. Chromatographic run is within 20 minutes. Only a double channel absorbance detector is needed, without the use of programmable absorbance detector, diode-array detector or gradient elution. It will be a useful tool to study the role of carotenoids, vitamins A and E in human chronic diseases such as coronary heart disease and cancer.

INTRODUCTION

Carotenoids, vitamins A and E have been postulated to play an important role in human chronic diseases such as cancer and coronary heart disease (1-3). Simultaneous high-performance liquid chromatographic (HPLC) analyses for these vitamins usually need gradient elution (4-6), a programmable UV detector or an expensive photodiode array detector (5-9). Alternatively, carotenoids and vitamins A/E are analyzed by different mobile phase systems (10). Nelis and De Leeheer's procedure (11) that can analyze at least 5 individual carotenoids simultaneously in the serum is currently one of the best for carotenoid analysis.

Here, we extended Nelis and De Leeheer's procedure by monitoring at an extra wavelength of 280 nm, and use of α-tocopheryl acetate as internal standard (IS) to include retinol and α-tocopherol with carotenoid analysis in a single chromatographic run.

MATERIALS AND METHODS

A) Reagents/Standards

Retinol, α-tocopherol and α-tocopheryl acetate (IS) were obtained from Sigma Chemical Co. (St. Louis, MO) and prepared in absolute ethanol. The actual concentration of each standard was determined spectrophotometrically using absorptivity values (1%, 1cm): retinol=1780 at 325 nm, α-tocopherol=75.8 at 292 nm, and α-tocopheryl acetate= 43.6 at 285 nm (12). Hexane and components of mobile phase (described below) were of HPLC grade.

B) HPLC system

The HPLC system (Waters) included an U6K injector, an M6000A solvent delivery system, an M400 absorbance detector (436/280 nm

for sample analysis and 313/280 nm for peak identification), an 820 Maxima work station, a Nova-Pak C-18 column (250x3.9 mm) and a Nova-pak guard column. Carotenoids were detected at 436 nm and vitamins A and E at 280 nm, which is the best filter for our system to detect both retinol and α-tocopherol. Mobile phase is acetonitrile: methylene chloride: methanol (7:2:1), degassed by vacuum for 30 minutes and pumping at 1.0 mL/min flow rate. All analyses were performed in ambient temperature.

C) Sample preparation

Serum (150 uL) was deproteinized with 150 uLs of ethanol containing echinenone and α-tocopheryl acetate as internal standards. After vortexing for 10-20 seconds, 400 uLs of hexane were added. The mixture was vortexed again for 30-40 seconds to extract retinol, tocopherols and carotenoids and centrifuged at 3000 rpm for 10 minutes to separate the phase. Upper layer (hexane) was aspirated to another tube, dried under nitrogen and reconstituted with 100 uL mobile phase. Finally, 80 uLs were injected to HPLC for analysis.

C) Qualitative analysis

Six serum samples were randomly selected and prepared without adding tocopheryl acetate internal standard to check any endogenous interfering peaks which might coelute with tocopheryl acetate. Peak identification was validated with retention time, spiked test and 313/280 nm ratio response.

D) Quantitative analysis

Within-run precision was evaluated by analyzing a randomly chosen serum sample 10 times and between-run precision was calculated from values obtained from a serum sample (chosen for

quality control) which was included in each batch analysis. Recovery was studied by adding both vitamins A and E to a serum sample and the amount obtained from the experiment (difference of spiked and unspiked samples) was compared to the actual amount added. Three calibration standards containing vitamins A (0.22-1.8 ug/mL) and E (5.8-46.5 ug/mL) were analyzed to evaluate linearity. Serum samples collected from twenty patients undergoing angiographic examinations were analyzed with this procedure (I) and Bieri's procedure (II, reference 12) for vitamins A and E in the comparison study. Linear regression and correlation coefficient were analyzed by Statistix software version 4.0 (Analytical Software, St. Paul, MN).

RESULTS AND DISCUSSION

A) Qualitative analysis

Since α-tocopheryl acetate is a widely used IS for the analyses of retinol (9), tocopherols (8,9,12) and carotenoids (9), it is chosen in this study. All six samples without adding α-tocopheryl acetate (IS) were not found to have any apparent peaks at retention time of α-tocopheryl acetate, validating its use as an IS. Both retention time and spiked test confirmed the identity of retinol A and α-tocopherol peaks. Signal ratios (peak height) at 313 nm/280 nm for vitamins A/E peaks from standard and serum samples are shown in Table 1. Close agreement of signal ratio between standard and samples for vitamins A (2.71 vs 2.66) and E (0.0947 vs 0.0862) further validate the peak identity.

B) Quantitative analysis

The coefficients of variation (CV) of within-run precision (n=10) for both vitamins were below 5% and those of between-run precision (n=9) were below 8% (Table 2). These are comparable to

Table 1. Signal ratios at 313nm/280nm of vitamins A/E peaks from standard and serum samples

	A (std)	A (serum)	E (std)	E (serum)
N	2	6	2	6
Mean	2.71	2.66	0.0947	0.0862
Minimum	2.63	2.55	0.0942	0.0803
Maximum	2.79	2.81	0.0952	0.0909

A (std): signal ratio for vitamin A peak from standard.
A (serum): signal ratio for vitamin A peak from serum sample.
E (std): signal ratio for vitamin E peak from standard.
E (serum): signal ratio for vitamin E peak from serum sample.

Table 2. Within-run and between-run precision

Precision	N	Mean, ug/mL	Standard deviation, ug/mL	Coefficient of variation, %
Vitamin A				
Within-run	10	0.573	0.0268	4.7
Between-run	9	0.618	0.0452	7.3
Vitamin E				
Within-run	10	6.21	0.178	2.9
Between-run	9	11.7	0.596	5.1

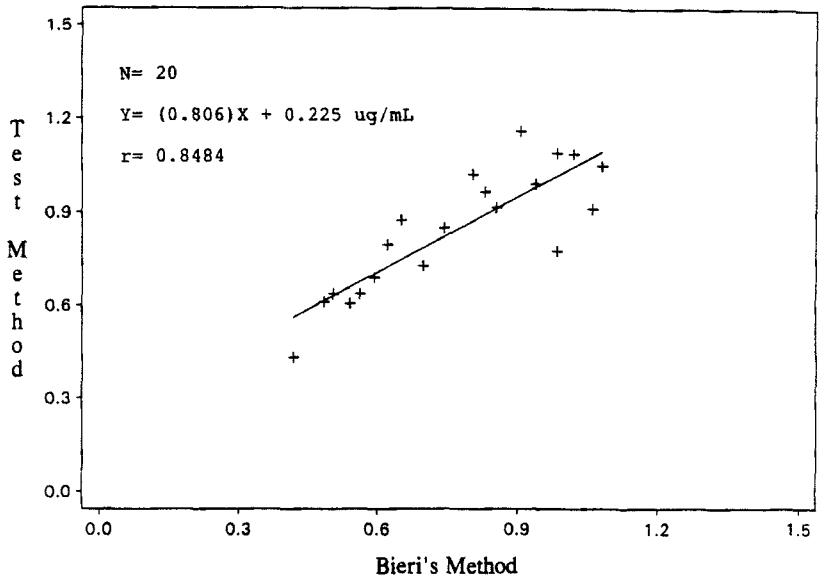

Figure 1. Comparison study of vitamin A (n=20).

other simultaneous procedures. Recoveries were 118% and 111% for vitamins A and E respectively. Analytical linearities were up to at least 1.8 ug/mL (vitamin A) and 46.5 ug/mL (vitamin E).

Comparison studies are shown in Figures 1 and 2 and chromatograms monitored at both 436 nm and 280 nm are shown in Figure 3. Retinol, α-tocopherol and α-tocopheryl acetate peaks are resolved and the run is within 10 minutes for vitamins A/E and 20 minutes for carotenoids, which is similar to other isocratic procedures (5,8) using similar column length. But the run time is shorter than gradient procedures (5,6) and total analysis time is more economic than running carotenoids and vitamins A/E separately (10). A minor peak between α-tocopherol and α-tocopheryl acetate was found in about 20% (8 out of 40) of samples and its retention time is very sensitive to variation in the components of the

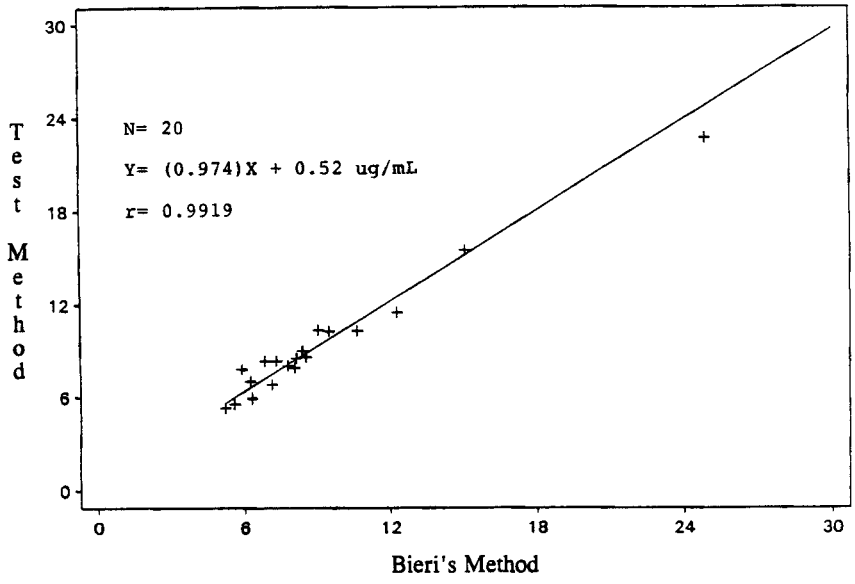

Figure 2. Comparison study of vitamin E (n=20).

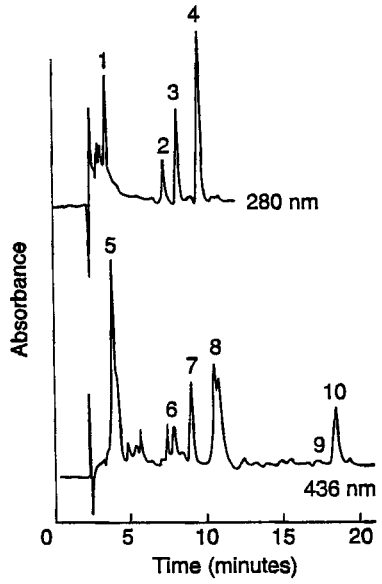

Figure 3. Chromatogram of a typical serum sample. Peak identification: 1=retinol, 2=Γ-tocopherol, 3=α-tocopherol, 4=α-tocopheryl acetate, 5=lutein and zeaxanthin, 6=cryptoxanthin, 7=echinenone, 8=lycopene, and 9=α-carotene, 10=ß-carotene.

mobile phase. Based on retention times of peaks of standard and samples, gamma-tocopherol was also separated by this system. Most simultaneous procedures (5-7,9) used a programmable UV/VIS detector or a photodiode array detector monitoring at three wavelengths of maximal absorbance for retinol, α-tocopherol and carotenoids to obtain their maximal sensitivity. One wavelength (280 or 292 nm) can be used to detect both retinol and α-tocopherol at the compromise of maximal sensitivity (4,8). This procedure only requires a double channel absorbance detector (280 nm and 436 nm) without the use of programmable UV/VIS detector, expensive diode-array detector or gradient elution.

Therefore, an isocratic carotenoid procedure was successfully extended to a procedure that can analyze carotenoids and vitamins A and E in a single run simultaneously. It is simple and more economic in total analysis time, reagents and equipment than many procedures commonly used at the present time. This simultaneous procedure will facilitate the study of roles of carotenoids, vitamins A and E in human research.

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