Simultaneous Determination of Vitamins A and E and Carotenoids in Plasma by Reversed-Phase HPLC in Elderly and Younger Subjects

Zahur Zaman, Peter Fielden, and Peter G. Frost

A reversed-phase high-performance liquid-chromatographic method for the simultaneous determination of retinol, α-tocopherol, α-carotene, β-carotene, cryptoxanthin, lutein/zeaxanthin, and lycopene is described. This method was applied to plasma measurements in healthy young and elderly subjects. The plasma, deproteinized with ethanol, is extracted twice with n-hexane. After evaporation, the residue is dissolved in 50 μL of tetrahydrofuran and made up to 200 μL with ethanol. Samples (50 μL) are injected onto a 250 × 4.6 mm column of 5-μm-particle Spherisorb ODS1 (Phase Separations) that had been equilibrated with solvent mixture A:B (90:10 by vol) [A = 100 mmol/L ammonium acetate in methanol: acetonitrile (80:20 by vol) and B = 100 mmol/L ammonium acetate in water] at 2 mL/min. The analytes are eluted by running a 12-min linear gradient to 100% A; solvent A is then maintained for 10 min. Intrabatch CVs were 2.3%, 3.3%, 2.8%, 3.6%, 3.6%, and 3.0% for retinol, α-tocopherol, lutein/zeaxanthin, cryptoxanthin, lycopene, and β-carotene, respectively. The corresponding interbatch CVs were 4.9%, 5.8%, 12.3%, 6.5%, 8.0%, and 3.4%.

Indexing Terms: retinol · tocopherol · lutein · zeaxanthin · cryptoxanthin · lycopene · carotene · age-related differences

Currently there is a considerable clinical interest in the inverse relation between a cancer risk and the plasma concentrations of β-carotene, retinoids, and vitamin E (1). The protective effect of these compounds may be related to their antioxidant properties. However, emphasis on β-carotene to the exclusion of other carotenoids found in common dietary constituents may be misplaced. Thompson et al. (2) reported 20 regularly occurring carotenoids in Canadian subjects. Of these, five, namely, α- and β-carotenes, cryptoxanthin, lutein, and lycopene, were the main components, with β-carotene accounting for 14% of the total. All these carotenoids can quench oxygen-derived free radicals (3), but only α- and β-carotenes and cryptoxanthin have provitamin A activity (4).

Retinoic acid, an oxidation metabolite of retinol (vitamin A), can sustain all biological actions of retinol except with regard to vision and reproduction (5). The beneficial effects of retinoic acid on proliferation and differentiation have already been applied therapeutically in the treatment of dermatoses, e.g., cystic acne (6), and the compound is also being tested for treatment of malignancies (7, 8).

These new developments in the functions of retinoids, carotenoids, and vitamin E suggest that assessment of these compounds should be carried out more rigorously than has hitherto been the case. Several high-performance liquid-chromatographic (HPLC) methods have been developed for determining retinoic acid and retinol, separately or together (9, 10), and for determining retinol, α-tocopherol (11), and the carotenoids together (12–15). In our hands these latter methods (12–15) gave irreproducible results. The method described here has good reliability characteristics; we used it to quantify retinol, α-tocopherol (vitamin E), lutein/zeaxanthin, cryptoxanthin, lycopene, and β-carotene in 20 elderly subjects and 20 younger subjects.

Materials and Methods

Materials

Retinol, retinyl acetate, α-tocopherol, and lycopene were purchased from Sigma Chemical Co. (Poole, Dorset, UK). β-Carotene, lutein, β-cryptoxanthin, and echinone were a generous gift from Roche Products (Welwyn Garden City, Herts, UK). We purchased 15-mL polypropylene conical centrifuge tubes with caps from Alpha Labs. (Eastleigh, Hamps, UK), Millipore filters from Millipore (Bedford, MA), HPLC columns from Phase Separations (Clywd, Wales, UK), and the LDC ultraviolet-visible absorbance detector from LDC Analytical (Stone, Staffs, UK). Three (low, medium, and high) lyophilized standard reference materials (SRM 968a) were acquired from the National Institute of Standards and Technology (NIST, Gaithersburg, MD).

Methods

Chromatography. The chromatographic system consisted of Spectra Physics (SP; Hemel Hempstead, UK) HPLC apparatus Model SP8700 XR, SP8780 autosampler, SP4200 dual integrator, and SP8440 and LDC de-
tectors. The main column (250 × 4.6 mm) and the guard
column (50 × 4.6 mm) were packed with 5-μm particles of
Spherisorb ODS1 (Phase Separations). All mobile-
phase solvents were prefiltered through 0.45-μm Milli-
pore filter discs (cat. no. HVLP 04700 for hydrophilic
solvents, HATF 0.47 EP for hydrophobic solvents). Be-
fore use, all solvents were purged with purified helium
gas for 30–40 min; the gas was also allowed to bubble
through the solvents during the run.
The solvents were (A) 100 mmol/L ammonium acetate
in methanol:acetonitrile (80:20 by vol) mixture and (B)
100 mmol/L ammonium acetate in water. After presequilibration with solvent mixture A:B (90:10 by
vol), a 12-min linear gradient was run to 100% A and
maintained for 10 min. This was followed by resequili-
bration over 5 min to the initial conditions. Pro-
grammed wavelength changes to 325 nm at 2 min be-
fore injection, 292 nm at 7.5 min after injection, and 450
nm at 13 min enabled selective monitoring of retinol,
α-tocopherol, and the carotenoids, respectively. The run
time from injection to injection was 27 min. The chro-
matography was performed at ambient temperature.
Sample handling. All handling and experimental pro-
cedures were carried out in a darkened room. Blood
samples were placed and transported in specially pro-
vided lighttight containers. Plasma from blood collected
into heparin solution and (or) serum fractions were
stored in plastic tubes under N2 at −70 or −20 °C. Any
sample that needed to be kept longer than a week was
stored at −70 °C.
Sample extraction. We mixed 0.5 mL of serum or
plasma and an equal volume of ethanol containing re-
廷yl acetate (4.27 μmol/L) and echinone (0.31 μmol/L)
as internal standards in a 15-mL polypropylene conical
centrifuge tube. After rotamixing these components for
30 s, we added 2 mL of n-hexane, securely capped the
tube, further rotamixing for 30 s, and then centrifuged
the sample at 2000 × g for 2–3 min. The organic super-
natant layer was removed into a 12 × 75 mm glass tube
and the aqueous layer was reextracted with another 2
mL of n-hexane. The combined organic phase was eva-
aporated under N2, and the residue was carefully disso-
volved in 50 μL of tetrahydrofuran (THF) and made up to
200 μL with ethanol. We applied 50–μL fractions to the
HPLC column by using an autoinjector. The method is
amenable to proportional reduction in scale.
Sample stability. Sera or plasma samples from
healthy volunteers were pooled and aliquoted into four
groups (n = 4 for each group) of 3-mL fractions. Samples
in group I were extracted and analyzed without delay.
The remainder underwent the following treatments:
Group II was frozen under N2 at −20 °C and subjected
to four cycles of freezing and thawing; freezing time varied
between 20 and 24 h. Groups III and IV were stored
under N2 at room temperature for 24 h and for 7 days,
respectively.
Analytical recovery experiments. Pooled human
plasma or serum samples were supplemented with four
concentrations of retinol, α-tocopherol, lutein, crypt-
oxanthin, lycopene, and β-carotene. The volume of the
supplement solution was kept low (10–20 μL) so as not
to cause protein precipitation. These samples (n = 4 for
each concentration) were extracted as above.
Three lyophilized standard reference materials from
NIST were also processed to determine the concentra-
tions of retinol, α-tocopherol, and β-carotene.
Internal standards and calibration curves. Retinyl ac-
etate was used as an internal standard for retinol and
α-tocopherol. Echinone, a synthetic carotenoid, served
as an internal standard for lutein/zeaxanthin, lycopene, cryptoxanthin, and α- and β-carotene.
Stock solutions of individual standards were prepared
in methylene chloride. Before measuring the concen-
trations, methylene chloride was evaporated under N2
but never to complete dryness. The residue was dissolved in
excess n-hexane (for carotenoids) or ethanol (for retinol,
retinyl acetate, and α-tocopherol) to give absorbances of
0.10 to 0.50 at relevant wavelengths. The concentra-
tions were calculated by using HPLC purities and ab-
sorptivities. The absorptivity values chosen for 10 g/L
solutions in 1-cm-pathlength cells were: echinone,
ε2370 (16); retinyl acetate ε292 (17); and
those cited in references 4 and 18.
Samples for calibration curves were prepared by add-
ing known amounts of standards to human serum (19)
and processing them as above. The calibration curves
were constructed by plotting integrated peak-area ra-
tios vs concentrations. At least three concentrations
were used for each analyte.
Quality-control material. Pooled normal human se-
rum was supplemented with analytes and stored in
2-mL aliquots under N2 at −70 or −20 °C. Each tube
was used only once after thawing. Each run included
quality-control samples.
Results and Discussion
We tried previously published mobile-phase mixtures
(11–16) but obtained the best results under the condi-
tions described here. Addition of ammonium acetate to
the mobile phase gave sharper peaks. Selective moni-
toring of all the analytes via a single detector requires
programmed wavelength changes at 325, 292, and 450
nm. Various modifications of the gradient were made to
obtain adequate separation for these wavelength
changes to occur at baseline. Unfortunately, the sepa-
ration of lutein and α-tocopherol was insufficient to en-
able the necessary wavelength change from 292 nm to
450 nm. Therefore, to quantify all of the compounds in
a single run, we used two detectors coupled to a dual-
channel recording integrator. Figure 1 shows chromato-
grams of a standard mixture. Because we were unable to
get pure zeaxanthin standard, we do not know whether
the latter would coelute with lutein. Figure 2 depicts
results of plasma extracts from two patients and a
healthy adult. One patient (Figure 2A) was treating
herself with garlic pills, a heart tonic made from haw-
thorn berries, and “Natuur” tablets; her total plasma
carotenoids were 8.20 μmol/L, with α-carotene (0.89
μmol/L) and β-carotene (3.50 μmol/L) accounting for
~54% of the total. The other patient (Figure 2B), with
hepatic failure due to cryptogenic cirrhosis, had low plasma concentrations of vitamin A and carotenoids; the plasma concentration of vitamin E (34.59 μmol/L) was within the normal range.

In all our subjects, young or old, γ-tocopherol (Figure 2, peak 3') was never >10–11% of α-tocopherol. In most cases, it was much less than that.

Monitoring extracts of human sera at 450 nm showed several peaks that, as yet, we have been unable to identify.

Solubility and extraction. Unlike retinol and α-tocopherol, carotenoids were only moderately soluble in ethanol, methanol, and acetonitrile at room temperature. At lower temperatures (e.g., −20 °C), carotenoids precipitated out and did not re dissolve when returned to room temperature. Therefore, we prepared all the stock solutions in methylene chloride and made the working solutions by diluting the stock solutions with ethanol. A further complication arose when reconstituting the residue after evaporating the hexane extract to dryness. Small volumes of mobile phase A—with or without ammonium acetate, ethanol, methanol, or acetonitrile—did not re dissolve the residue completely, particularly the carotenoids. Methylene chloride, chloroform, and THF gave 100% redissolution but distorted the peak shapes of retinol, retinyl acetate, and α-tocopherol. As a successful compromise, we carefully dissolved the residue in 50 μL of THF and made up the solution to 200 μL with ethanol. Some workers (15) have used ultrasonication to redissolve the residue in ethanol.

The effect of methylene chloride, chloroform, and THF on peak shapes has been ascribed to complex interactions caused by dissimilarities between polarities and solubility properties of the injection and mobile-phase solvents (20).

Recovery and linearity. A single hexane extraction recovered 100% of retinol and α-tocopherol; 92–95% of lutein, cryptoxanthin, and echinenone (results not shown); but at best only 80% of α- and β-carotenes and lycopene. This differential behavior of carotenoids to n-hexane extraction has been described by Barua et al. (21). This also explains why inclusion of an internal standard (echinenone), which did not behave identically with respect to all the carotenoids, failed to correct for differences in the efficiency of the first hexane extraction. A second extraction with hexane improved the extraction to 100% (Table 1). Therefore, we used throughout a procedure involving two n-hexane extractions. Our recoveries for carotenoids after the first hexane extraction were similar to that reported by MacCrehan and Schonberger,

![Fig. 1. Chromatograms of a standard mixture of retinol, α-tocopherol, and carotenoids](image.png)

**Table 1. Recovery of Vitamins A and E and β-Carotene from Human Plasma by n-Hexane Extraction**

<table>
<thead>
<tr>
<th>Added</th>
<th>Mean recovered (n = 4)</th>
<th>% recovery (±SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 × extr.</td>
<td>2 × extr.</td>
</tr>
<tr>
<td>Retinol</td>
<td></td>
<td></td>
</tr>
<tr>
<td>7.895</td>
<td>8.026</td>
<td>8.038</td>
</tr>
<tr>
<td>3.648</td>
<td>4.000</td>
<td>3.986</td>
</tr>
<tr>
<td>1.874</td>
<td>1.986</td>
<td>1.998</td>
</tr>
<tr>
<td>0.987</td>
<td>0.977</td>
<td>0.984</td>
</tr>
<tr>
<td>Vitamin E</td>
<td></td>
<td></td>
</tr>
<tr>
<td>219.41</td>
<td>199.66</td>
<td>214.56</td>
</tr>
<tr>
<td>108.71</td>
<td>98.65</td>
<td>107.73</td>
</tr>
<tr>
<td>54.85</td>
<td>51.22</td>
<td>54.84</td>
</tr>
<tr>
<td>27.43</td>
<td>24.95</td>
<td>27.70</td>
</tr>
<tr>
<td>β-Carotene</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3.319</td>
<td>2.621</td>
<td>3.300</td>
</tr>
<tr>
<td>1.659</td>
<td>1.179</td>
<td>1.835</td>
</tr>
<tr>
<td>0.830</td>
<td>0.638</td>
<td>0.833</td>
</tr>
<tr>
<td>0.415</td>
<td>0.352</td>
<td>0.415</td>
</tr>
</tbody>
</table>

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Fig. 2. Chromatograms from a self-medicating patient (A), a patient with a liver cirrhosis (B), and a healthy subject (C).

Upper panel (A1, B1, and C1): Output of channel 2 monitored continuously at 450 nm for carotenoids. The peaks and retention times (min) are: 1, retinol (4.35); 2, retinyl acetate (5.81); 3', α-tocopherol (10.09); 3, α-tocopherol (11.78); 4, cryptoxanthin (13.98); 5, echinonene (15.10); 6, lycopene (17.33); 7, β-carotene (18.80); 8, β-carotene (19.46); 9, lutein/cryptoxanthin (6.46). Detector responses at 0.024 (A1, B1, and C1) and 0.014 (A2, B2, and C2) full-scale deflection.

(15) but poorer than those of others (13, 14). Those groups performed their recovery experiments by mixing equal volumes of ethanol containing the desired analytes and plasma. We, however, have tried to mimic physiological conditions by first mixing 0.5 mL of plasma with 10-20 μL of an ethanolic solution of the desired analyte and then extracting as described in Methods. Our final extraction recoveries of lutein and lycopene were better than those reported by Cantilena and Nierenberg (22). There was no significant difference in recoveries from plasma or serum samples, as has also been found by other workers (13). Our results for the standard reference materials compared well with those certified by NIST (Table 2).

The calibration curve for the method was linear to concentrations of at least 10.26 μmol/L for retinol, 237 μmol/L for α-tocopherol, 5.44 μmol/L for β-carotene, 4.46 μmol/L for lutein, 4.59 μmol/L for cryptoxanthin, and 4.51 μmol/L for lycopene.

Detection limits. The detection limit was defined as the lowest concentration of an analyte in a standard solution that could be detected from zero with 95% confidence (n = 10). The minimum detectable concentrations (μmol/L) were: retinol, 0.35; α-tocopherol, 5.80; lycopene, 0.28; cryptoxanthin, 0.13; lutein, 0.12; and α- and β-carotene, all 0.13. However, using the more sensitive detector (LDC SM 400) improved the detection limit 10-fold.

Intra- and interbatch imprecision. Intrabatch imprecision, calculated from the differences in duplicate determinations, and interbatch imprecision, calculated from the quality-control samples placed in each run, are

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Low</th>
<th>Medium</th>
<th>High</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Our results(a)</td>
<td>NIST(b)</td>
<td>Our results(a)</td>
</tr>
<tr>
<td>Retinol</td>
<td>0.69</td>
<td>0.042 ± 0.056</td>
<td>1.79</td>
</tr>
<tr>
<td>α-Tocopherol</td>
<td>11.05</td>
<td>10.31 ± 0.51</td>
<td>25.01</td>
</tr>
<tr>
<td>β-Carotene</td>
<td>0.57</td>
<td>0.497 ± 0.147</td>
<td>1.77</td>
</tr>
</tbody>
</table>

\(a\) Mean of two results.

\(b\) Concentration certified by NIST, mean ± SD.
### Table 3. Imprecision

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Interbatch (n = 24)</th>
<th>Intrabatch (n = 20)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Conc, μmol/L</td>
<td>CV, %</td>
</tr>
<tr>
<td>Retinol</td>
<td>2.007</td>
<td>0.098</td>
</tr>
<tr>
<td>Vitamin E</td>
<td>26.31</td>
<td>5.76</td>
</tr>
<tr>
<td>Lutein/zeaxanthin</td>
<td>0.241</td>
<td>0.030</td>
</tr>
<tr>
<td>Cryptoxanthin</td>
<td>0.138</td>
<td>0.008</td>
</tr>
<tr>
<td>Lycopene</td>
<td>0.235</td>
<td>0.032</td>
</tr>
<tr>
<td>β-Carotene</td>
<td>1.695</td>
<td>0.058</td>
</tr>
</tbody>
</table>

* Calculated from differences of duplicates.

### Table 4. Intrabatch Drift

<table>
<thead>
<tr>
<th>Position in assay run</th>
<th>Beginning</th>
<th>Middle</th>
<th>End</th>
</tr>
</thead>
<tbody>
<tr>
<td>Retinol</td>
<td>2.169*</td>
<td>2.150</td>
<td>2.164</td>
</tr>
<tr>
<td>Vitamin E</td>
<td>32.13</td>
<td>32.62</td>
<td>32.04</td>
</tr>
<tr>
<td>Lutein/zeaxanthin</td>
<td>0.480</td>
<td>0.475</td>
<td>0.478</td>
</tr>
<tr>
<td>Cryptoxanthin</td>
<td>0.742</td>
<td>0.756</td>
<td>0.717</td>
</tr>
<tr>
<td>Lycopene</td>
<td>1.119</td>
<td>1.108</td>
<td>1.136</td>
</tr>
<tr>
<td>β-Carotene</td>
<td>1.421</td>
<td>1.410</td>
<td>1.410</td>
</tr>
</tbody>
</table>

* Concentration, μmol/L.

shown in Table 3. Our interbatch precision was much better and our intrabatch precision was either comparable with (for retinol, α-tocopherol, and cryptoxanthin) or better than (for lycopene and β-carotene) that of Thurnham et al. (14). The between-day performance of the Cantilena and Nierenberg method (22) was better than our interbatch precision, but our intrabatch precision was better than their within-day precision. Intrabatch drift was estimated by placing one of the standard samples at the beginning, middle, and end of a batch and observing differences in its concentration. The batch size varied between 25 and 45 samples. The results (Table 4) of a typical run show that there is no significant drift during a run.

Sample stability. In specimens protected from light and stored anaerobically, retinol, α-tocopherol, and carotenoids did not undergo any significant degradation after repeated freezing and thawing or storage at room temperature for 7 days (Table 5). Similar results have been reported by others for freezing and thawing (23) and for storage at room temperature for 24 h (16).

### Plasma concentrations in elderly and young subjects.

Plasma concentrations of retinol, α-tocopherol, β-carotene, cryptoxanthin, lutein/zeaxanthin, and lycopene were determined in two group of subjects. One group comprised 20 healthy men and women (10 of each sex) of mean age 36 years (range 23–55). The second group consisted of 20 healthy elderly subjects of equal sex mix with a mean age of 80 years (range 77–89). The results (Table 6) for retinol, α-tocopherol, and β-carotene for the younger subjects are similar to those reported for US adults (13, 15, 24). The mean concentrations of α-tocopherol and β-carotene were higher in the elderly group. This may reflect an age-related decrease in the circulating clearance of retinol and lipoproteins (25, 26), which transport carotenoids and other lipid-soluble compounds.

In conclusion, the method described gives excellent recoveries and improved resolution of retinol, α-tocopherol, lutein/zeaxanthin, cryptoxanthin, lycopene, and α- and β-carotenes. As reported elsewhere, we have used

### Table 5. Stability of Vitamins A and E and Carotenoids in Human Plasma

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Untreated sample</th>
<th>After freezing and thawing</th>
<th>After storage at room temp</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1.120 ± 0.028*</td>
<td>1.110 ± 0.031</td>
<td>1.113 ± 0.035</td>
</tr>
<tr>
<td>Retinol</td>
<td>35.04 ± 0.580</td>
<td>34.73 ± 0.569</td>
<td>34.62 ± 0.580</td>
</tr>
<tr>
<td>Vitamin E</td>
<td>0.318 ± 0.009</td>
<td>0.318 ± 0.012</td>
<td>0.315 ± 0.009</td>
</tr>
<tr>
<td>Lutein/zeaxanthin</td>
<td>0.247 ± 0.005</td>
<td>0.239 ± 0.007</td>
<td>0.239 ± 0.006</td>
</tr>
<tr>
<td>Cryptoxanthin</td>
<td>0.657 ± 0.009</td>
<td>0.654 ± 0.009</td>
<td>0.652 ± 0.019</td>
</tr>
<tr>
<td>Lycopene</td>
<td>0.525 ± 0.009</td>
<td>0.525 ± 0.009</td>
<td>0.523 ± 0.011</td>
</tr>
</tbody>
</table>

* Results are mean ± SEM (μmol/L) of 4 determinations each.

### Table 6. Plasma Concentrations of Retinol, Vitamin E, and Carotenoids (μmol/L) in Elderly and Young Subjects

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Young (n = 20)</th>
<th>Elderly (n = 20)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>Range</td>
</tr>
<tr>
<td>Retinol</td>
<td>2.283</td>
<td>0.408</td>
</tr>
<tr>
<td>Vitamin E</td>
<td>23.22</td>
<td>5.387</td>
</tr>
<tr>
<td>Lutein/zeaxanthin</td>
<td>&lt;0.123-0.309</td>
<td>&lt;0.123-0.309</td>
</tr>
<tr>
<td>Cryptoxanthin</td>
<td>&lt;0.127-0.467</td>
<td>&lt;0.127-0.467</td>
</tr>
<tr>
<td>Lycopene</td>
<td>&lt;0.279-0.996</td>
<td>&lt;0.279-0.996</td>
</tr>
<tr>
<td>β-Carotene</td>
<td>0.464</td>
<td>0.324</td>
</tr>
</tbody>
</table>

* Many values for these analytes were below detection limits so no statistical analysis was performed.
this method to investigate plasma concentrations of these analytes in patients with Alzheimer disease and multi-infarct dementia (27, 28).

References