Measurement of Dietary Carotenoid Isomers Using HPLC-ECD

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Key Words
Vitamin A, Carotenoids, HPLC-ECD, Tissue

Summary
This application note describes an HPLC-ECD method using a C30 stationary phase column for the analysis of isomeric forms of carotenoids in animal and plant tissues.

Introduction
Much of the interest in dietary carotenoids, exclusive of their pro-vitamin A activity, is related to their possible actions as preventive agents in diseases associated with oxidative stress. These electron-rich compounds can act as antioxidants *in vitro* and their possible role of protection from reactive oxygen and nitrogen species *in vivo* has received much attention.[1]

Dietary carotenoids can each be found as a variable mixture of geometric and positional isomers (e.g., all *trans*, *cis*-9-, and *cis*-13) – See Figure 1. These isomers may occur naturally or can be formed during processing[2] and show a variety of biological properties and chemical activities. For example, β-carotene isomers exhibit significant differences in pro-vitamin A activity,[3] tissue distribution[4] and detector responsivity.[5]

Many reversed phase HPLC methods that utilize monomeric bonded C18 stationary phases for carotenoid analysis are incapable of completely separating the various isomers and in some instances, may lead to inaccurate assessment of nutritional and other health-related properties.[6,7,8] HPLC methods that utilize a silica-based C30 stationary phase specifically designed for carotenoid separations have shown significant, and often superior, overall enhancement in shape selectivity for a variety of carotenoid isomers.[5,6,7] Photodiode array (PDA) detection in combination with C30 separations represents an extremely useful technique for carotenoid analysis. This approach, however, has limited sensitivity when analyzing the lower levels present in serum and animal tissue, particularly for the less abundant isomers.[7] Electrochemical detection (ECD) is a highly sensitive form of HPLC detection for lipid soluble vitamins and antioxidants.[9,10,11] The coulometric array is a multichannel form of electrochemical detection that allows resolution of compounds based on differences in voltammetric behavior and qualitative characterization of peaks even at trace levels.[12,13,14] The basis of detection and resolution using coulometric array relates to differences between analytes in their ability to delocalize charge. Since the antioxidant properties of a compound are closely related to these same structural characteristics, the application of coulometric array detection to carotenoid analysis may provide some additional biochemical insight.[15]

This application note examines the use of coulometric array detection with HPLC using a C30 stationary phase for analysis of isomeric forms of carotenoids in animal and plant tissues. HPLC coulometric array ECD can be used both as a complementary approach to traditional methods and to provide unique information on carotenoids.

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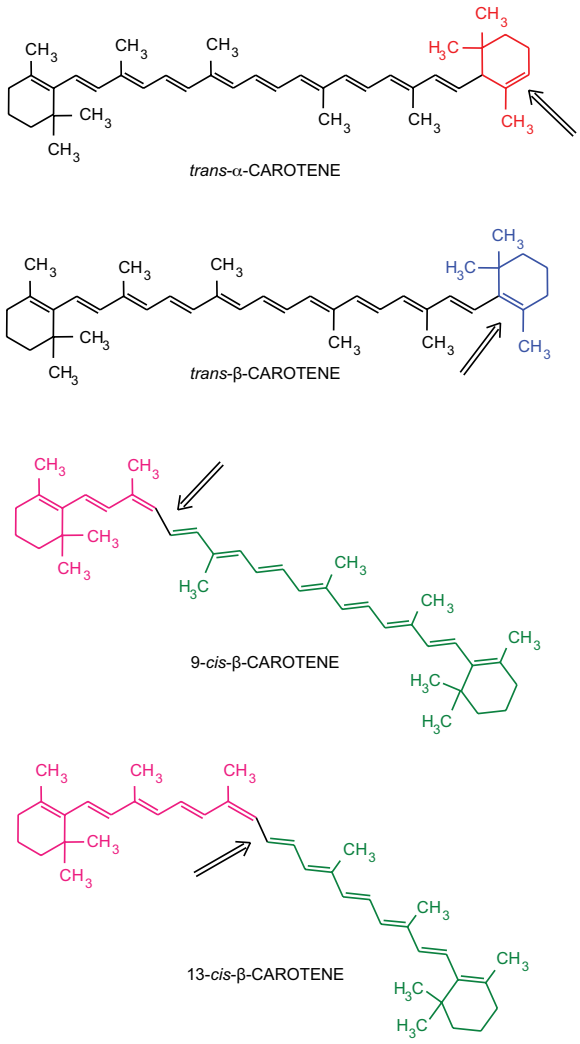

Figure 1. The structure of α- and ß-carotene and two geomrtic isomers of ß-Carotene.

Materials and Methods

The isocratic analytical system consisted of a pump, an autosampler, a thermostatic chamber, a twelve-channel Thermo Scientific™ Dionex™ CoulArray™ Coulometric Array Detector and a UV/vis detector placed prior to the array.

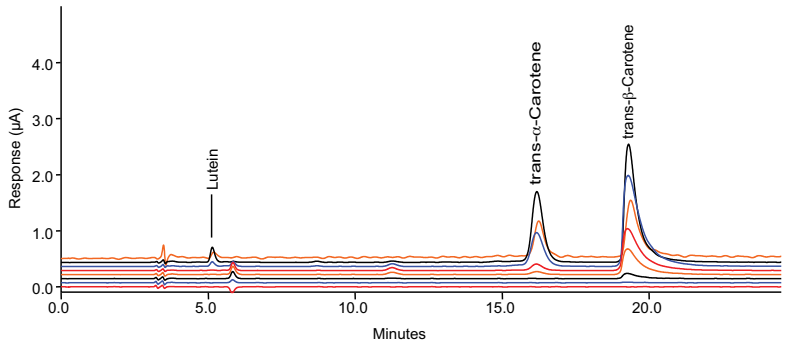

Figure 2. Analysis of a raw carrot extract.

Conditions	
LC	
Column:	C30, 5 µm, 4.6 × 250 mm
Mobile Phase:	Methanol – Methyl-*tert*-butyl Ether (MTBE) – 1.0M Ammonium Acetate, pH 4.4 (63:35:2) (v/v/v)
Flow Rate:	1.0 mL/min
Temperature:	28 °C
Injection Volume:	10 µL
Detector	
Electrochemical Detector:	Model 5600A, CoulArray
Applied Potentials:	100, 160, 220, 280, 340, 400, 460 and 520 mV vs. Pd.
Detector Wavelength:	450 nm

Standards

Standards were prepared by dissolving ~1 mg/10 mL chloroform followed by dilution in 10 mL ethanol. Solutions were assayed spectrophotometrically and assigned a concentration value based on molar absorbtivity. Butylated hydroxyanisole was then added as a preservative. Dilutions were made in mobile phase.

Sample Preparation

A 0.5 mL volume of serum or standard was mixed with 0.5 mL ethanol/10 mg/L BHA. After mixing for 1 min, 1.5 mL of hexane was added and after mixing for an additional 10 min. was centrifuged (4,000 rpm, 10 min). Approximately 1.0 mL of supernatant was withdrawn and the remaining sample extracted with an additional 1.5 mL of hexane. Combined hexane extracts were evaporated to dryness under a stream of nitrogen. Finally, the residue was dissolved in 0.25 mL of mobile phase.

Results and Discussion

The separation of carotenoid isomers was completed within 25 minutes (Figure 2). For coulometric array ECD, the assay was linear over the range of 0.1 to 500ng on column. The limit of detection (lod) was ~20 pg (s/n 3:1) for both trans-α-carotene and trans-β-carotene; a twenty fold improvement over UV detection. The within-run peak height response variability (% relative standard deviation, %R.S.D.) was 0.42 and 0.45 for 10ng of trans-α and trans-β-carotene, respectively (n=8). For 200 pg, an amount well below the LOD for absorbance detection, EC response variability was 3.57 and 6.45 %R.S.D. for trans-α and trans-β-carotene, respectively (n=7).

Figure 3 illustrates HPLC-coulometric array profiles of MTBE extracts of a raw (Figure 3A) and a thermally processed carrot (Figure 3B). Peak elution profiles are similar to those commonly obtained using absorbance detection where isomeric forms of α- and β-carotene are apparent as a result of thermal processing. Figure 4 shows a representative chromatogram of a human serum extract. Levels of *trans* isomers, estimated using external standards, showed good agreement between EC and absorbance (±3%). The output from 4 electrochemical channels (to illustrate voltammetric response ratios) along with absorbance at 450 nm are shown. Response ratios between adjacent channels when compared to those obtained from standards (Figure 2) are in close agreement. This is indicative of high peak purity for both *trans*-α and *trans*-β-carotene. The chromatogram in Figure 4 illustrates the ability to obtain useful qualitative information on low level analytes in a complex matrix. While the ability to detect and obtain spectral absorbance information is very limited, the EC response for a number of putative isomers is well above the lower limit of detection.

It is interesting to note that in Figures 3 and 4 the predominant EC response is obtained at 340mV for *trans*-β-carotene and 400 mV for *trans*-α-carotene (i.e. *trans*-β-carotene is more easily oxidized due to more effective hyper-conjugation). Furthermore, a peak eluting immediately prior to *trans*-α-carotene, which can be tentatively identified as 13-*cis*-β-carotene, has dominant response at the same channel as *trans*-β-carotene.[7] Other possible *cis* isomers, based on literature, may include the two unresolved peaks immediately prior to '13-*cis*-β-carotene' and possibly the 9-*cis*-β-carotene which elutes immediately after β-carotene.[7] This latter peak has an unique voltammetric profile with dominant response at 460 mV.

Conclusion

In conclusion, HPLC with a silica-based C30 stationary phase in combination with coulometric array electrochemical detection allowed selective measurement of carotenoids with an estimated 20-fold improvement in sensitivity (lod ca. 20 pg) as compared to UV-Vis absorbance detection. Response was linear over a 0.1 to 500 ng range. Qualitative voltammetric data obtained across serial coulometric sensors was used to aid in peak identification and provide information on the oxidation-reduction properties of analytes. The feasibility of detection and qualitative analysis of trace levels of *cis* isomeric forms of α and β-carotene in human serum has been demonstrated. This approach has recently been applied to measurement of isomeric forms of carotenoids in tissue.[16]

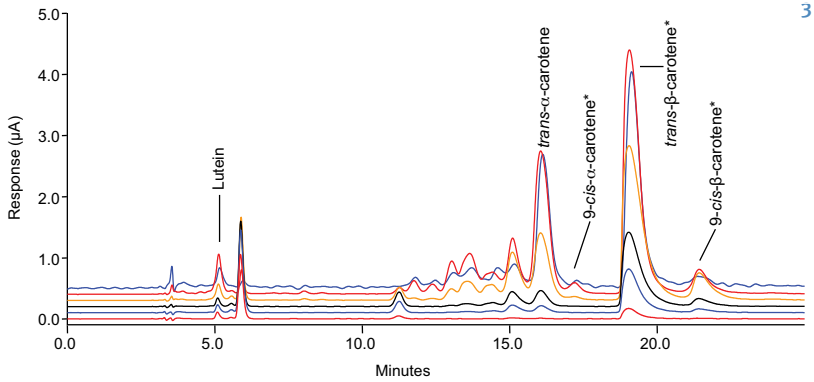

Figure 3. Analysis of a thermally processed carrot. (*tentative indentification).

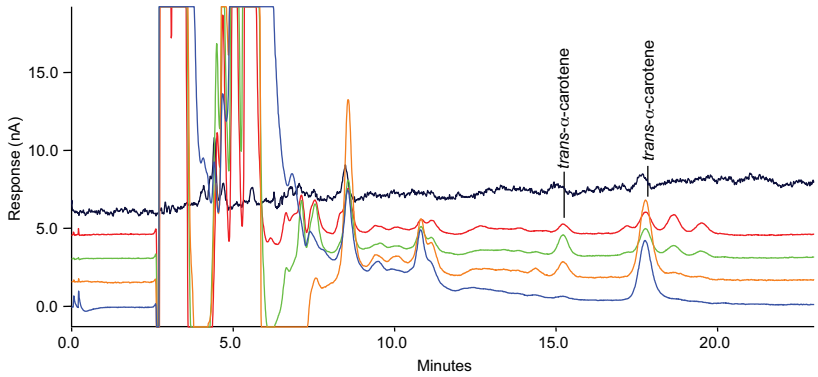

Figure 4. Low level serum.

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Ordering Information

Description	Part Number
HPG-3400RS Biocompatible Binary Rapid Separation Pump with Two Solvent Selector Valves	5040.0046
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