Carotenoid Analysis in Food and Drug Formulations

YMC AMERICA, INC.

Abstract

Interest in the perceived health benefits of carotenoids continues to require their separation and quantification. The minor shape difference among carotenoid isomers and the separation of individual species has been very challenging. Official carotenoid methods generally do not account for isomers that are biologically active. Published data on the vitamin A content of foods is inaccurate since not all the isomers are measured. To illustrate this problem, various foods are analyzed for their carotenoid isomer content. The carotenoid profiles for foods before and after exposure to high temperatures are shown.

This study employs the YMC Inc. Carotenoid™ column for reversed phase HPLC separations that is highly selective for the carotenoid geometric isomers. The utility of this tailored phase is demonstrated for the separation of carotenoids in foods, dietary supplements, pre-formulation concentrates and vitamin formulations.

Sample Preparation

Raw carrots
Approximately 4 grams of carrot were chopped and added to 5 mL of MTBE (methyl t-butyl ether). The MTBE was centrifuged to remove suspended solids and 20 µL of the supernatant was injected.

Canned (processed) carrots
Approximately 10 grams were chopped and added to 50 mL of MTBE. The MTBE was centrifuged to remove suspended solids and 20 µL of the supernatant was injected.

Collards
Approximately 10 grams of collard leaf were chopped and mixed with 10 mL of MTBE/MeOH (70:30). After sitting for 4 hours refrigerated, the solvent was centrifuged to remove suspended solids and 20 µL of the supernatant was injected.

Canned (processed) spinach
Approximately 10 grams of spinach were chopped and mixed with 10 mL of MTBE. After sitting for 4 hours refrigerated, the solvent was centrifuged to remove suspended solids and 20 µL of the supernatant was injected.

Tomato paste
Approximately 10 grams of tomato paste were mixed with 10 mL of MTBE. After sitting for 4 hours refrigerated, the solvent was centrifuged to remove suspended solids and 20 µL of the supernatant was injected.

Betatene®
Approximately 10 grams of Betatene were mixed with 10 mL of MTBE. After sitting for 4 hours refrigerated, the extract was centrifuged to remove suspended solids and 20 µL of the supernatant was injected.

Analysis Conditions

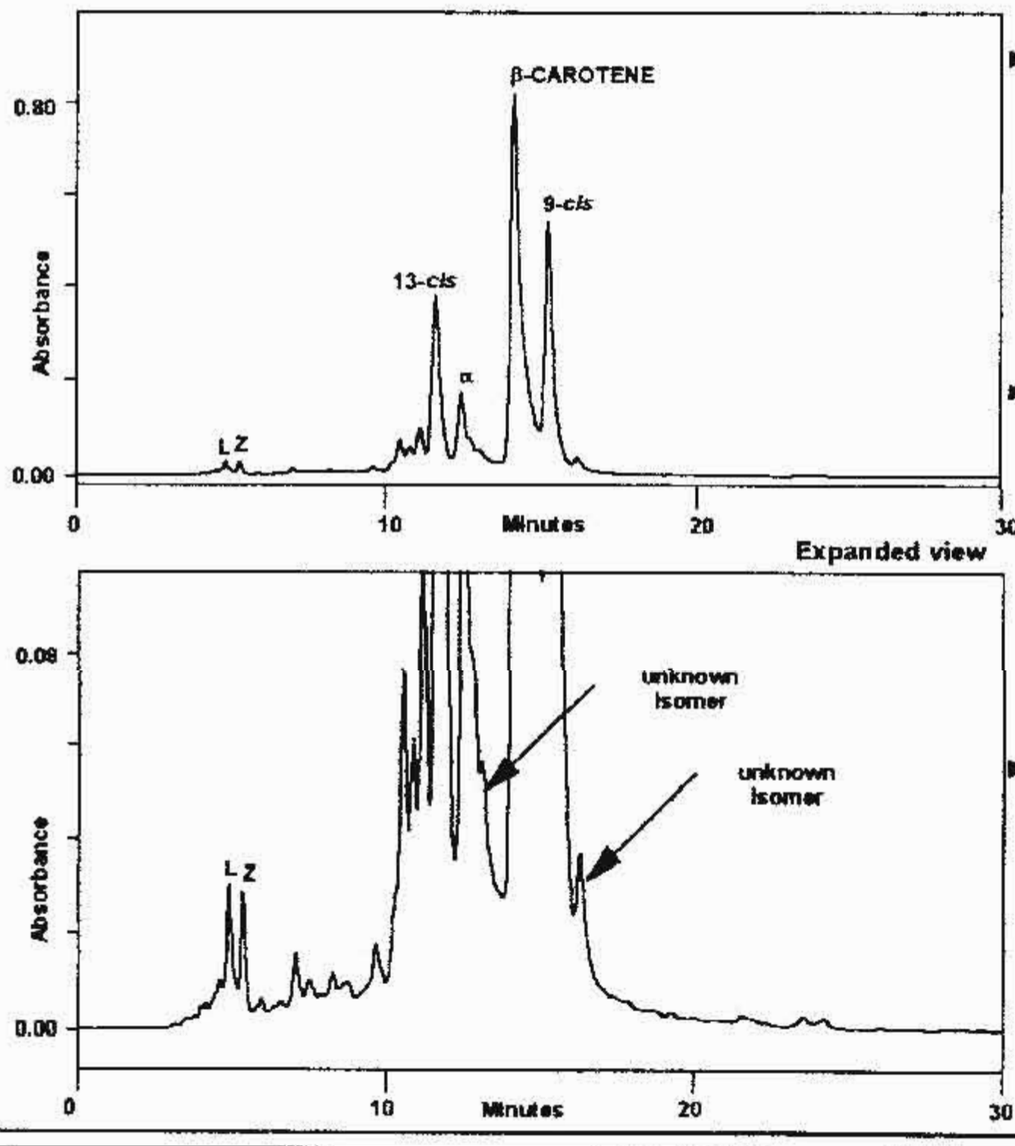
Analytical separation conditions were optimized for sample throughput. The original separation protocols using the Carotenoid column were developed by Dr. Lane Sander.[1] These separations were accomplished in 80 minutes using a 4.6 x 250 mm YMC, Inc. Carotenoid column. For this work the run time has been reduced to 30-40 minutes by modifying the gradient conditions

Column:	**YMC, Inc. Carotenoid 4.6 x 250 mm, 5 μm**
Mobile Phase:	**A=MeOH B=MTBE**
Gradient:	**30% B to 75% B in 40 minutes**
Flow rate:	**1.0 mL/min**
Detection:	**PDA (250 nm - 550 nm)**

- **Analysis resolution 4.8 nm**
- **Spectral resolution 1.2 nm**

1. Sander, et al. *Analytical Chemistry*, Vol. 66, No. 10, May 15, 1994 pp.1667-1674.

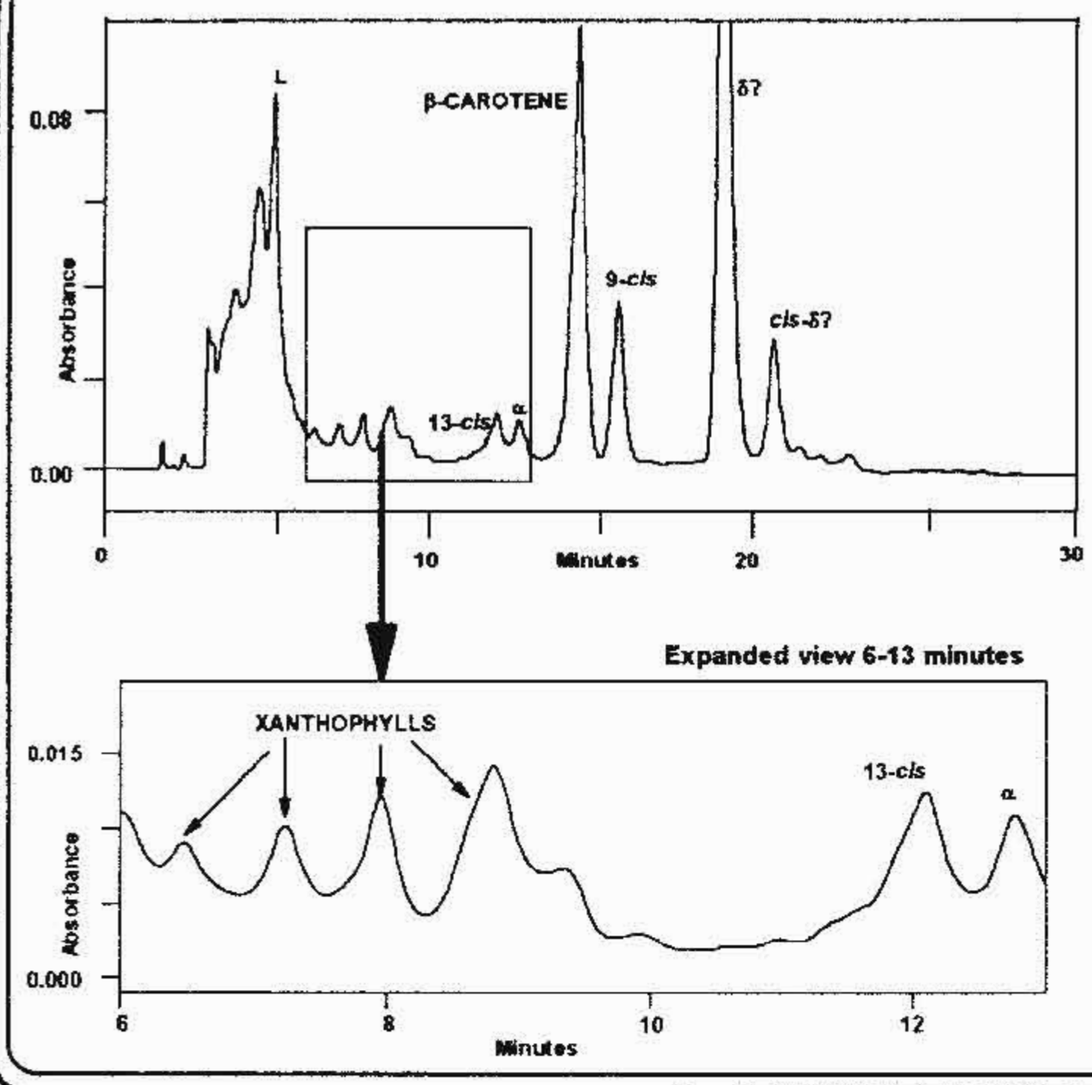
Analysis of Betatene



- **Betatene[2] is a commercially available mixture of carotenes including: lutein (L), zeaxanthin (Z), α-carotene (α) and β-carotene**
- **Analysis reveals large levels of 13-cis β-carotene (13-cis) and 9-cis β-carotene (9-cis). These isomers possibly formed during the processing of raw material**
- **Minor isomers are evident eluting after a-carotene and 9-cis β-carotene (→)**

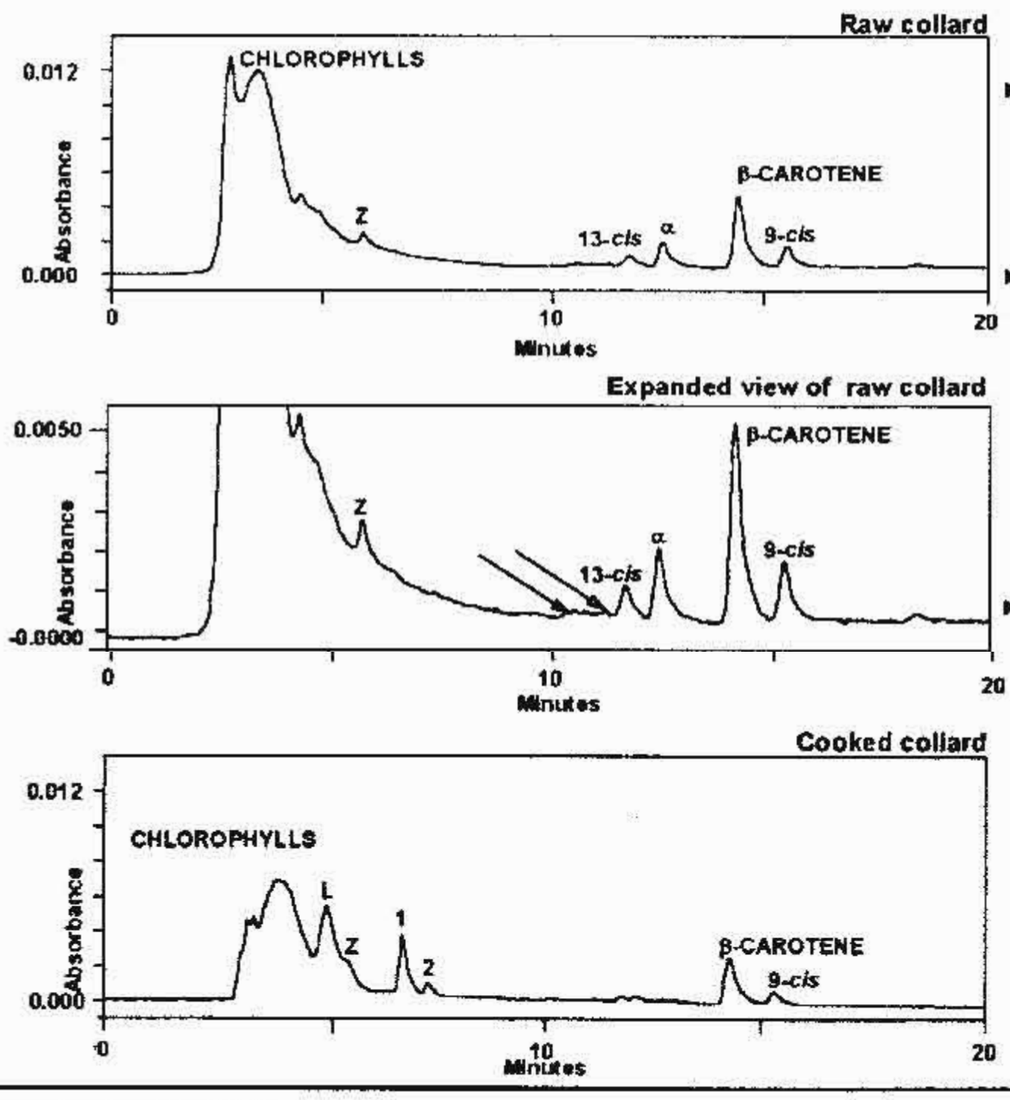
2. Betatene sample courtesy of Henkel Corporation

Analysis of Canned Spinach



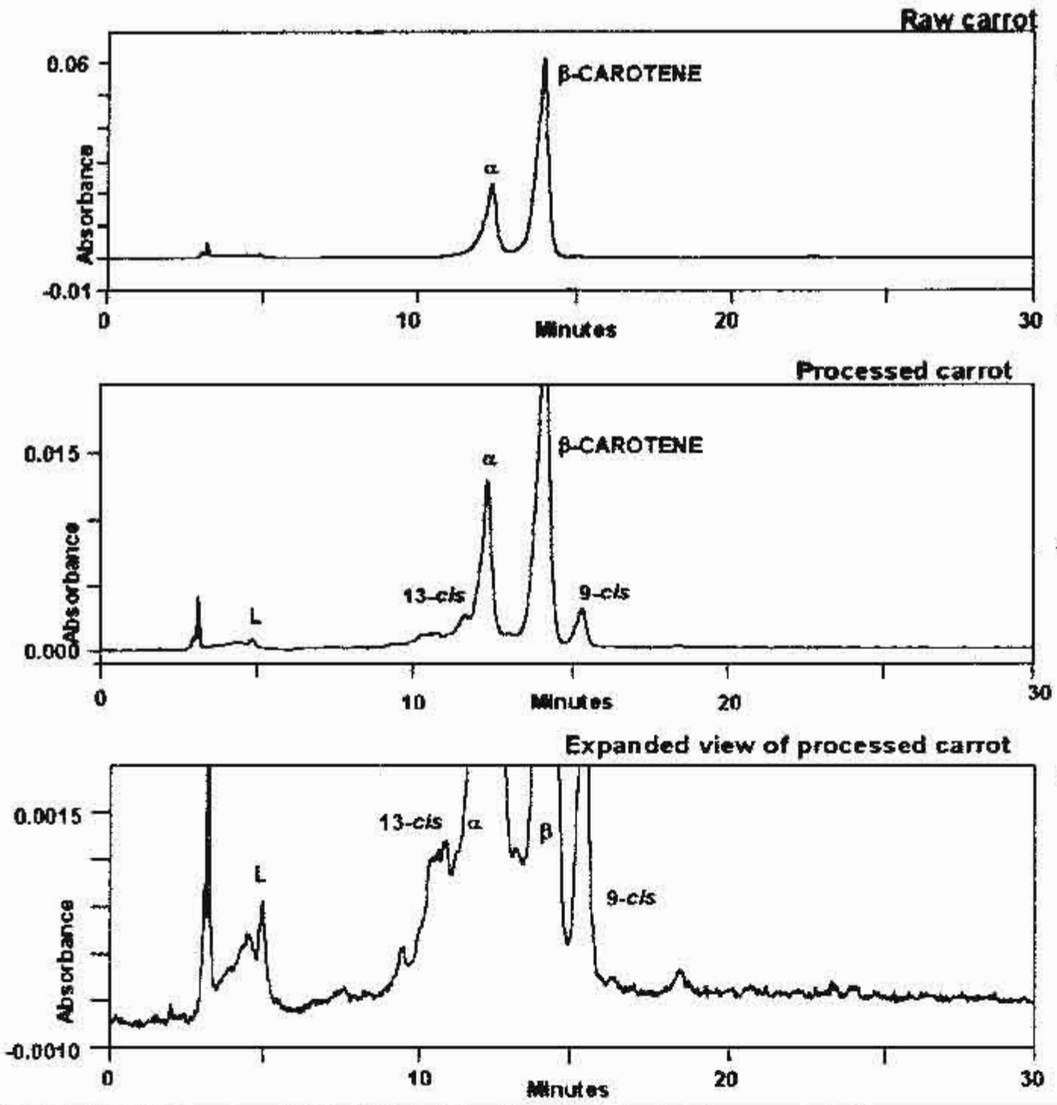
- Very high levels of lutein and very low levels of α-carotene evident in spinach
- A major peak elutes where δ-carotene (δ) is expected. No standard of δ-carotene was available
- Expanded view shows many xanthophyll related compounds with polar functional groups elute between 6 and 10 minutes
- Other carotene related compounds elute after δ-carotene

Comparison of Raw vs. Cooked Collards



- Very high chlorophyll levels evident in raw extract but reduced in cooked
- Minor carotene isomers (→) are eluting before 13-*cis* β-carotene. No standards available to positively identify them as 15-*cis* and di-*cis*
- Cooked collards show:
  - The formation of unknown xanthophylls (1, 2)
  - Reduction of 13-*cis* and α-carotene
  - Higher level of lutein (L) in cooked collards

Comparison of Raw and Processed Carrots

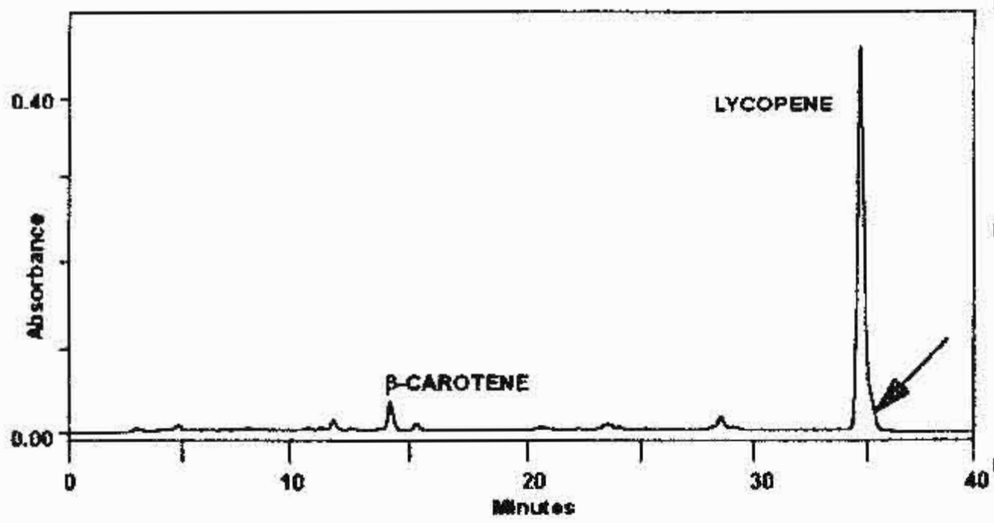


- Carotenoid profile in raw carrots is very simple, only α and β-carotene are present in large quantities
- Percentage of α-carotene (α) is 2x higher in canned (processed) carrots compared to the raw carrot
- Processed carrots also show higher levels of 13-*cis* β-carotene (13-*cis*) and 9-*cis* β-carotene (9-*cis*) isomers
- Lutein (L) level is elevated in processed carrot

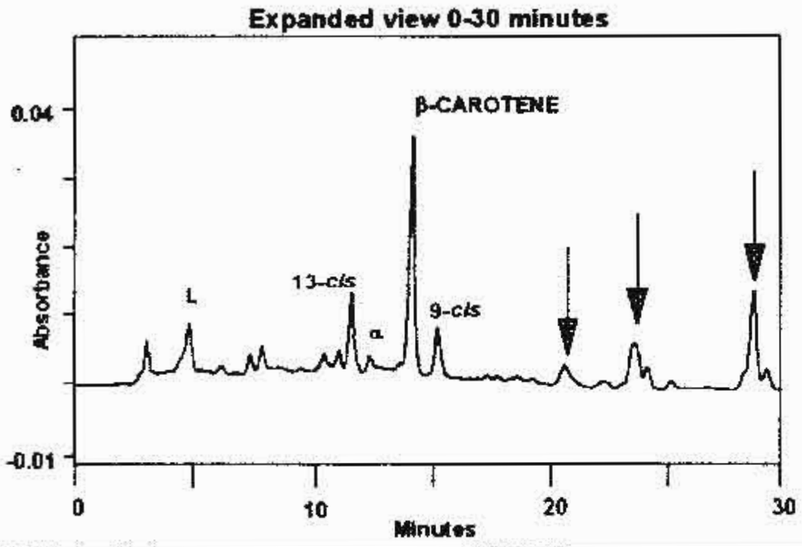
Comparison of Raw vs. Microwaved Carrot

- Ratio of α-carotene (α) to β-carotene is higher for microwaved carrot compared to raw carrot
- Microwaved carrot shows the appearance of 13-*cis* β-carotene (13-*cis*) and 9-*cis* β-carotene (9-*cis*) isomers
- Lutein (L) and zeaxanthin (Z) show minor changes after microwave cooking

Analysis of Tomato Paste

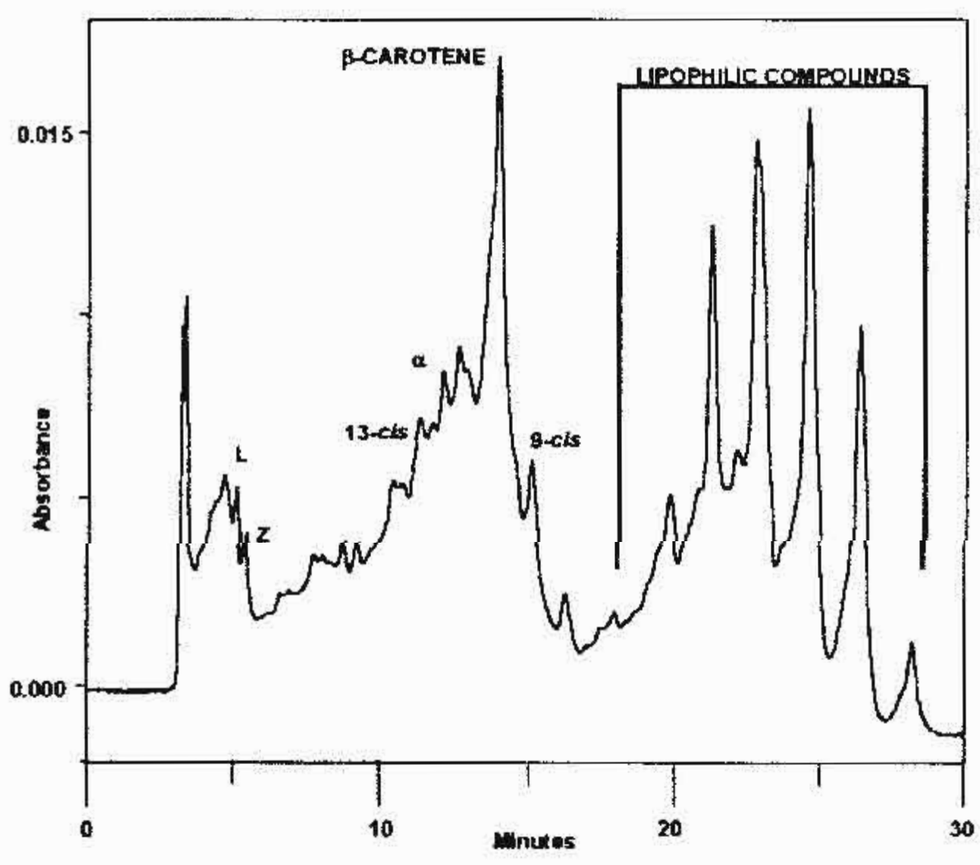


Expanded view 0-30 minutes



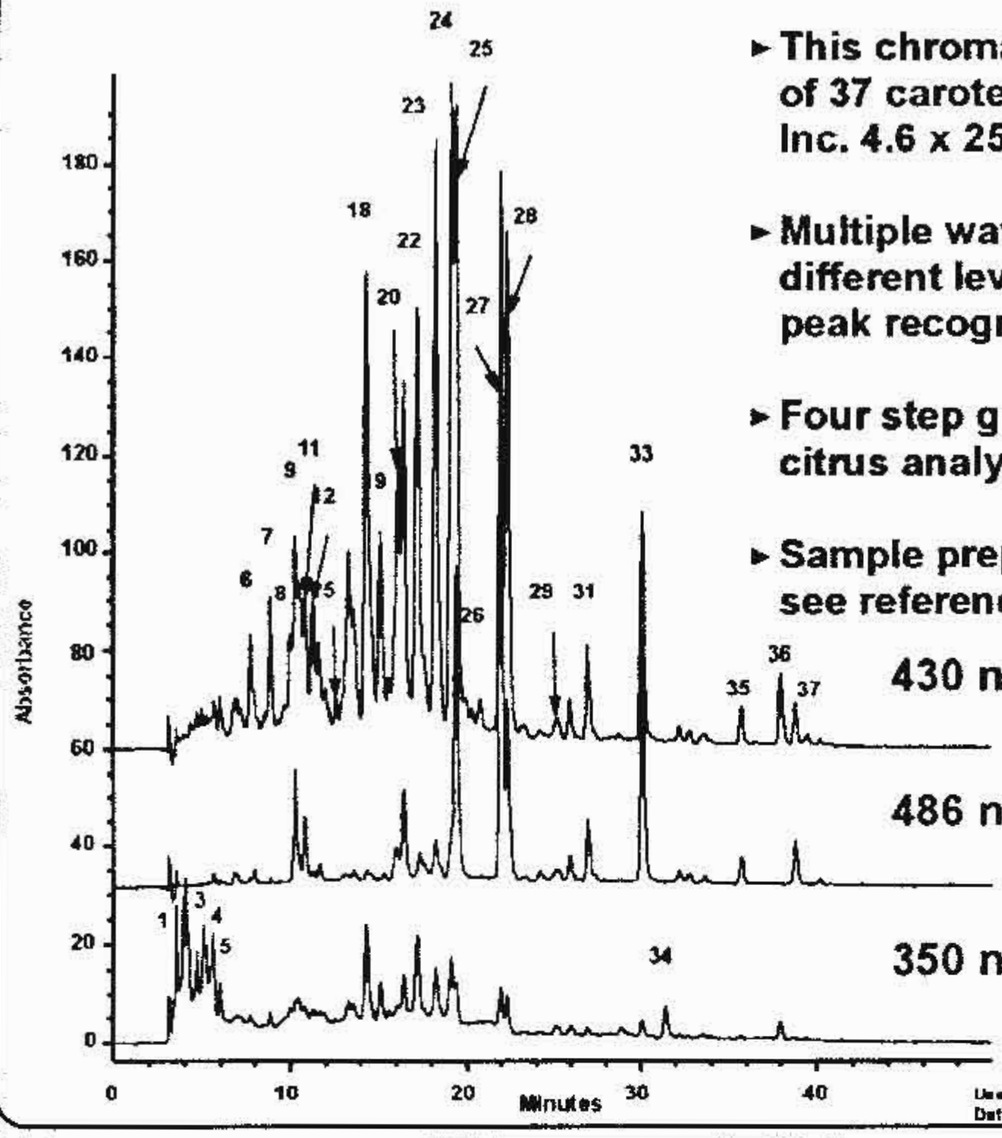
- Very high levels of lycopene are present which is indicated by the tomato's red color
- There is evidence that a lycopene isomer elutes after the lycopene peak at 35 minutes (→)
- Expanded view shows other major isomers include: lutein, 13-*cis* β-carotene (13-*cis*), β-carotene and 9-*cis* β-carotene (9-*cis*)
- Very low levels of α-carotene (α) in tomato paste
- Other unidentified isomers found at 21, 24 and 29 minutes (→)

Chili Pepper Extract



- Chili pepper extracted with 30:70 MeOH/MTBE
- Analysis shows many peaks, especially the lipophilic carotenoids and their potential esters
- Major isomers elute after α and β-carotene
- Saponification of the sample before extraction would simplify the chromatography

Analysis of Saponified Orange Juice



- This chromatogram clearly shows resolution of 37 carotenoid compounds on the YMC, Inc. 4.6 x 250 mm Carotenoid column
- Multiple wavelength detection provides different levels of sensitivity and enhances peak recognition
- Four step gradient optimized for difficult citrus analysis
- Sample preparation requires saponification, see reference below

Four Step Gradient Conditions

1) 90:5:5 MeOH/MTBE/H2O to 95:5 MeOH/MTBE in 12 minutes

2) 95:5 MeOH/MTBE to 86:14 MeOH/MTBE in 8 minutes

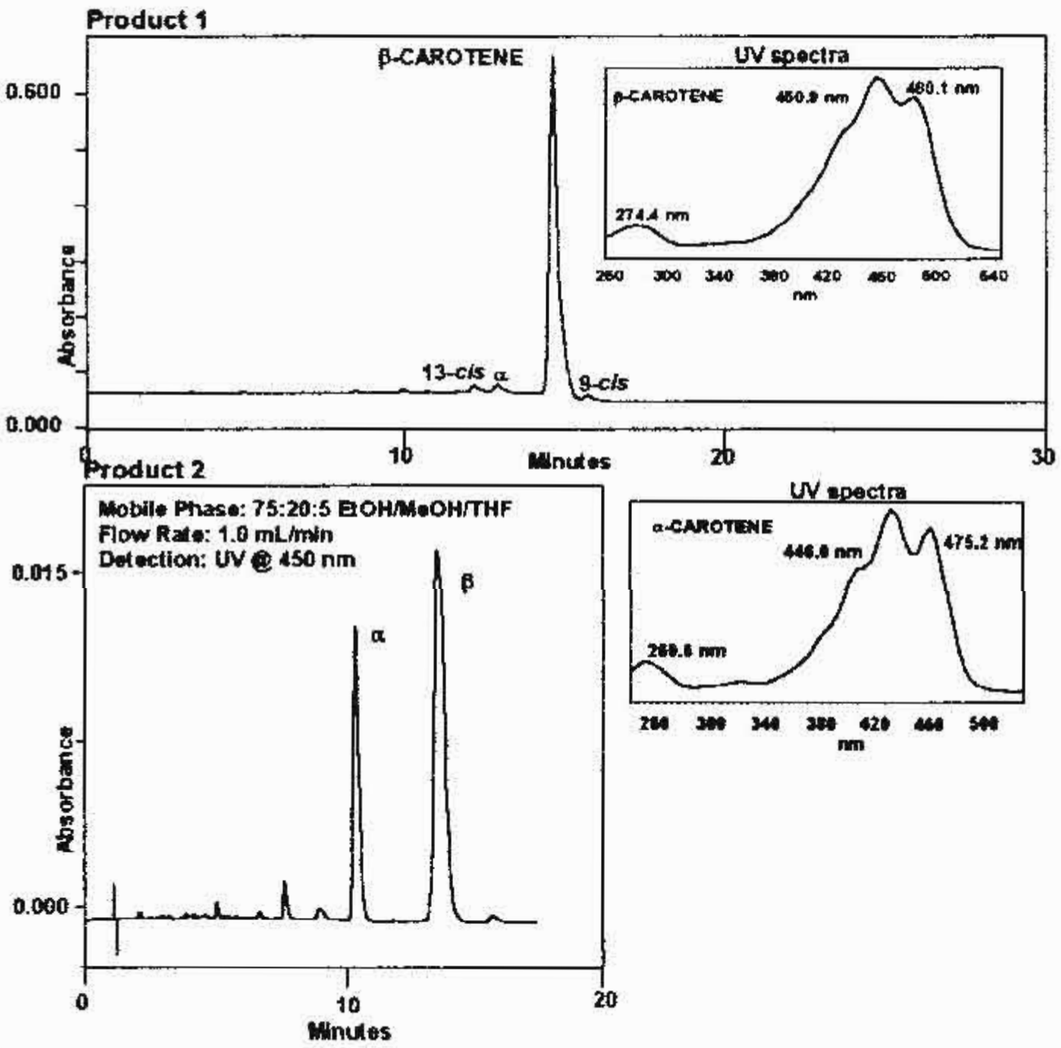
3) 86:14 MeOH/MTBE to 75:25 MeOH/MTBE in 10 minutes

4) 75:25 MeOH/MTBE to 50:50 MeOH/MTBE in 20 minutes

Flow rate: 1.0 mL/min

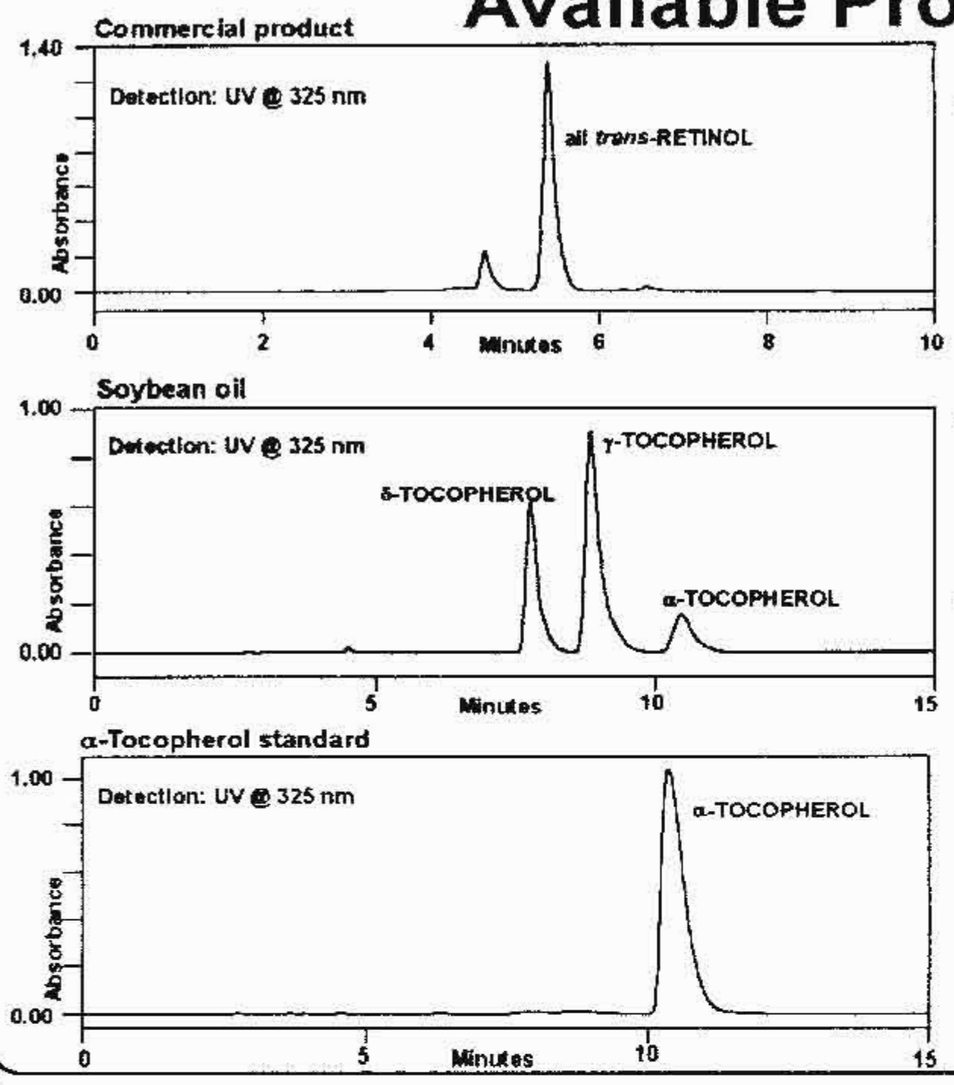
Used with permission of Dr. Russell Rouseff, University of Florida, CREC. Data published in *J. Agric. Food Chem.*, Vol 44, No. 8, 1996

Analysis of β-Carotene Vitamin Supplement Capsule



- Major differences exist between brands of β-carotene supplements
- A commercially available β-carotene supplement (product 1) shows relatively high purity
  - Typical impurities include α-carotene, 13-*cis* β-carotene and 9-*cis* β-carotene
- The second commercially available product shows 50% α and 50% β-carotene with other minor impurities at low levels

Vitamin Analysis of Commercially Available Products



- For more polar samples including vitamin A analogs, tocopherols and xanthophylls the samples are dissolved in MeOH
  - Polar samples not soluble in MTBE
- Analysis of all *trans*-retinol shows a few degradation products
- The YMC Carotenoid column resolves 3 common Vitamin E analogs from a mixture in soybean oil as well as an α-tocopherol standard

Mobile Phase: MeOH
Flow Rate: 1.0 mL/min

Isolation and Identification of Carotenoids

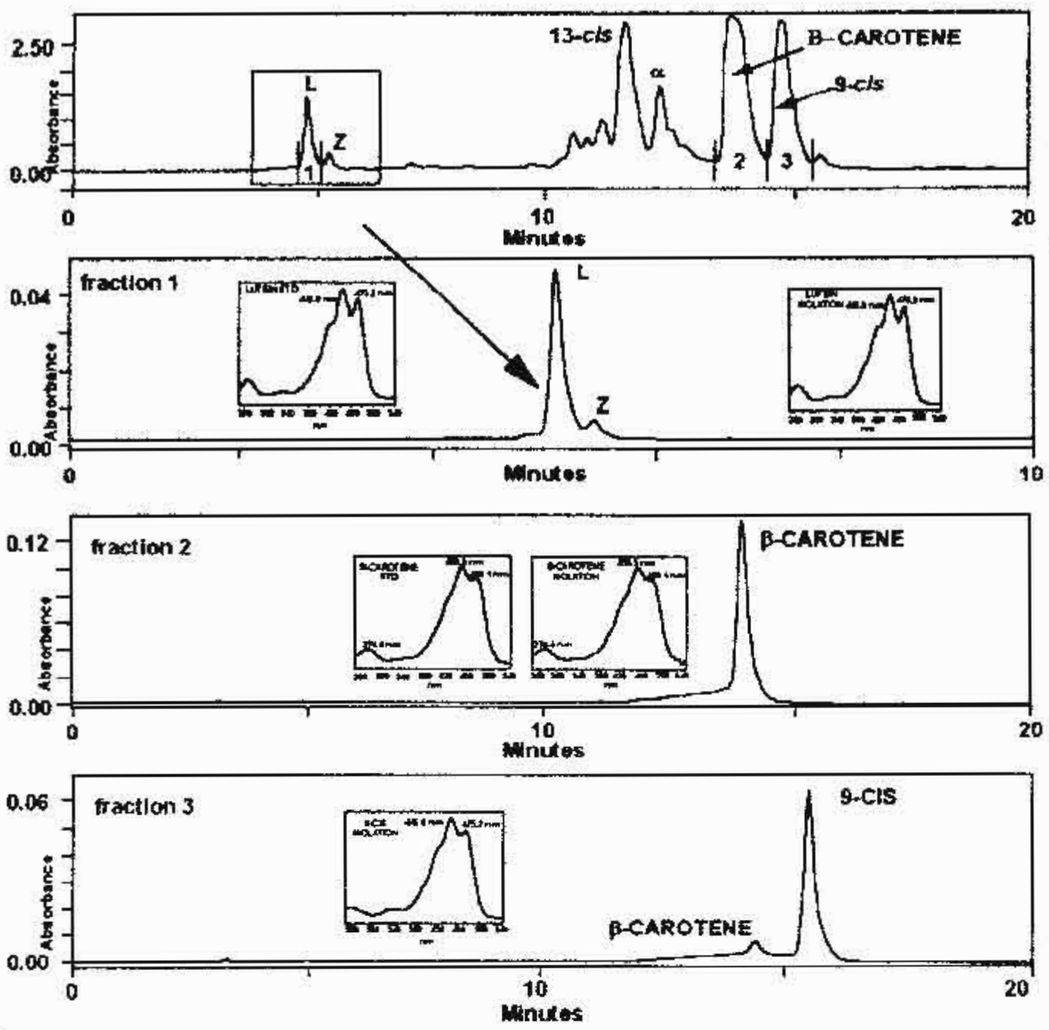
The YMC Carotenoid column is available in 3, 5 and 15 micron particle sizes for analytical and preparative separations.

It is useful and cost effective to isolate the carotenoid isomers in your lab because:

1. **Purchasing carotenoid standards is expensive**
2. **Degradation of isolated isomers is difficult to prevent**
3. **Many isomers are not commercially available**

The following panel shows the preparative separation of various carotenoid isomers from a natural product extract.

Isolation and Purification of Isomers from Betatene



- Preparative loadings of natural products yield pure carotenoid isomers
- This sample contained 4% lutein, 24% β-carotene and 18% 9-*cis* β-carotene before purification
- After purification:
  - Fraction 1 yields 90% pure lutein. Spectral comparison verifies peak identity
  - Fraction 2 yields 99% pure β-carotene. Spectral comparison verifies peak identity
  - Fraction 3 yields 93% pure 9-*cis* β-carotene. No standard was available to compare spectra. Higher purity is achievable by purifying this fraction again

Lutein standard courtesy of Kemin Industries

Conclusions

- The YMC, Inc. Carotenoid column is extremely versatile and selective for the analysis of carotenoids and fat soluble vitamins from many natural products
- Minimal sample preparation is required. Typically, only grinding or blending with MTBE and MeOH is required
- Simple mobile phases of MeOH and MTBE separate most isomers
- Dr. Rouseff's research combined the YMC, Inc. Carotenoid column and a multiple step gradient method to resolve 37 carotenoid isomers
- For very complex samples, saponification is required
- Analysis time can be optimized to isocratic conditions when the total profile is not required
- Processing foods through cooking (microwave, pressure cooking and heating) alters the carotenoid profile indicating formation of many cis isomers