

# HPLC Separation of All-*Trans*- $\beta$ -Carotene and Its Iodine-Induced Isomers Using a C30 Column

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## Key Words

Provitamin, Carotenoid, Antioxidant, Natural Product, Mass Spectrometry (MS), APCI

## Introduction

Carotenoids are a group of natural products that are common fat-soluble pigments in foods. Many therapeutic functions have been ascribed to these compounds, though the recognized role of  $\beta$ -carotene is as the main dietary source of vitamin A. Recently, protective effects of carotenoids against serious disorders such as cancer, heart disease and degenerative eye disease have been recognized.<sup>1</sup>  $\beta$ -Carotene is generally regarded as the most commercially important and widely utilized carotenoid. It is used as a food coloring agent, an antioxidant, and an important and safe provitamin A source. Most  $\beta$ -carotene is naturally present in the *trans* form; however, some amounts of the *cis* form of  $\beta$ -carotene are also present in foods.<sup>2</sup> Figure 1 shows the structures of all-*trans*- $\beta$ -carotene and its four important *cis* isomers, 15 or 15', 13 or 13', 11 or 11' and 9 or 9'. Using a typical reversed-phase column, such as a C18, it is difficult to separate *trans*- $\beta$ -carotene and its *cis* isomers.

Therefore a C30 stationary phase was applied to this separation challenge with the thought that the longer C30 alkyl chain of the stationary phase would be ideal for separating hydrophobic structurally related isomers.<sup>3</sup>

The work shown here describes an efficient and convenient way to acquire the isomers of  $\beta$ -carotene by exposing it to iodine in sunshine.<sup>4</sup>  $\beta$ -Carotene and its iodine-induced isomers were separated on a Thermo Scientific Acclaim C30 column, which is designed to provide high shape selectivity for separation of hydrophobic structurally related isomers and unique selectivity complementary to other reversed-phase columns (e.g., C18).<sup>5</sup> Seven compounds were identified as isomers of  $\beta$ -carotene, and the structures of four isomers were tentatively identified based on their chromatographic behaviors, spectral characters, and mass spectra obtained by a Thermo Scientific Dionex DAD-3000RS Rapid Separation Diode Array Detector and a Thermo Scientific MSQ Plus Mass Spectrometer.

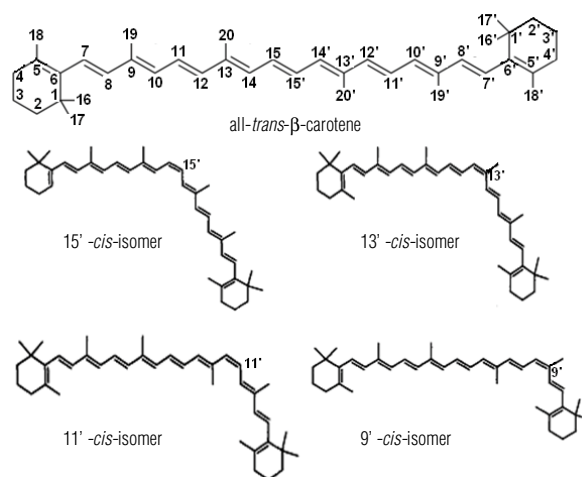


Figure 1. Structures of  $\beta$ -carotene and its four major *cis* isomers.

## Goal

To develop a better HPLC separation of  $\beta$ -carotene and its iodine-induced isomers than possible on a C18 reversed-phase column.

## Equipment

- Thermo Scientific Dionex UltiMate 3000 Rapid Separation Liquid Chromatography (RSLC) system, including:
  - SRD-3400 Integrated Solvent and Degasser Rack
  - LPG-3400RS Quaternary RS Pump
  - WPS-3000TRS Well Plate Sampler, Thermostatted
  - TCC-3000RS Thermostatted Column Compartment
  - DAD-3000RS Diode Array Detector (volume: 13  $\mu$ L; length: 10 mm; pressure limit: 120 bar)
- MSQ Plus™ Mass Spectrometer with APCI source
- Thermo Scientific Dionex Chromeleon Chromatography Data System (CDS) software, Version 6.80 SR9 or higher

## Reagents and Standards

Deionized (DI) water, 18.2 M $\Omega$ -cm resistivity

Acetonitrile (CH<sub>3</sub>CN), HPLC grade

(Cat.# AC610010040), Fisher Chemical

Methanol (CH<sub>3</sub>OH), HPLC grade (Cat.# AC610090040),  
Fisher Chemical

n-Hexane, HPLC grade (Cat.# H3025K-4),  
Fisher Chemical

Methyl tert-butyl ether (MTBE), HPLC grade  
(Cat.# BP2605-100), Fisher Chemical

All-*trans*- $\beta$ -carotene,  $\geq 97\%$ , Fluka Iodine,  $\geq 99.8\%$ ,  
Sigma-Aldrich

## Chromatographic Conditions

Analytical Column:	Acclaim™ C30, 5 $\mu$ m, 4.6 $\times$ 150 mm (P/N 075719)
Column Temperature:	10 °C
Mobile Phase:	MTBE/CH <sub>3</sub> CN/CH <sub>3</sub> OH In gradient (Table 1)
Flow Rate:	1.0 mL/min
Detection:	Absorbance at 475 nm
Injection Volume:	5 $\mu$ L

## MSQ-Plus Mass Detector Conditions

Ionization Interface:	APCI
Operating Mode:	Positive Scan
Scan Events:	Selected Ion Monitoring (SIM) scan: 537 m/z for all- <i>trans</i> - $\beta$ -carotene and its isomers
Probe Temperature:	400 °C
Corona:	30 $\mu$ A
Cone Voltage:	60 V
Nebulizer Gas:	Nitrogen at 60 psi

## Sample Preparation

### Solutions for Iodine Induction of Isomers

Solution 1 (I<sub>2</sub>-hexane, 1 mg/mL): dissolve 10 mg of I<sub>2</sub> in 10 mL of hexane in a brown-colored flask.

Solution 2 ( $\beta$ -carotene-hexane, 1 mg/mL): dissolve 20 mg of all-*trans*- $\beta$ -carotene standard in 20 mL of hexane in a brown-colored flask.

### Isomer Induction Procedure

Add 1 mL of Solution 1 and 20 mL of Solution 2 to a translucent flask. After shaking for 1 min, expose the sample to sunlight for 30 min, and then move the mixture to a brown colored flask. Preserve the sample in the dark.<sup>5</sup>

## Results and Discussion

### Separation of the Iodine-Induced

### *Trans*- $\beta$ -Carotene Solution

#### Selection of Mobile Phase

Nonaqueous reversed-phase (NARP) is usually used for determining fat-soluble compounds by HPLC so that the compounds are soluble throughout the analysis. A typical NARP mobile phase consists of a polar solvent (usually acetonitrile), a solvent with lower polarity (e.g., dichloromethane) to act as a solubilizer and to control retention by adjusting the solvent strength, and an amount of a third solvent with hydrogen-bonding capacity (e.g., methanol) to optimize selectivity.<sup>6</sup> Therefore, a mobile phase containing acetonitrile, methanol, and MTBE was used in this work to separate the fat-soluble  $\beta$ -carotene and its isomers.

#### Effect of Column Temperature

Preliminary experiments showed that column temperature significantly influenced the selectivity of C30 column for the separation of an iodine-induced all-*trans*- $\beta$ -carotene solution. Therefore, the effect of column temperature was evaluated further. Figure 2 shows the effect of temperature between 10 °C and 50 °C. The retention time decreased as expected when increasing the column temperature; however, lower column temperature provided better resolution. For example, while peak 2 and 3 could not be separated between 20 °C and 40 °C, a resolution (Rs) of 1.5 was achieved at 10 °C. There were three small peaks observed between peaks 1 and 2 at 20 °C, though only two of them could be found below 20 °C, and they coeluted with peaks 1, 2 and 3 when temperature was higher than 30 °C. Given that 10 °C provided the best resolution of the major peaks, it was used for all further studies.

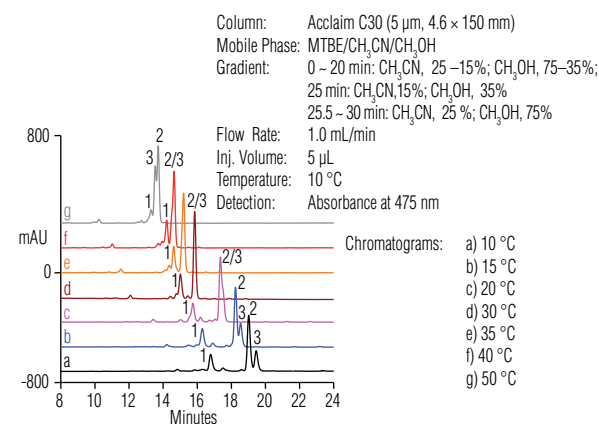


Figure 2. Chromatograms of an iodine-induced all-*trans*  $\beta$ -carotene solution on the Acclaim C30 column (5  $\mu$ m, 4.6  $\times$  150 mm) at different column temperatures.

Table 1. Gradient conditions for the separation of all-*trans*- $\beta$ -carotene and its iodine-induced isomers.

Time (min)	Mobile Phase A MTBE (% vol.)	Mobile Phase B CH <sub>3</sub> CN (% vol.)	Mobile Phase C CH <sub>3</sub> OH (% vol.)	Curve
0.0	0	25	75	—
20.0	50	15	35	5
25.0	50	15	35	5
25.5	0	25	75	5
30.0	0	25	75	5

## Comparison of the Separation on C18 and C30 Columns

Although the extensively-used C18 column is a good choice for the separation of low-polarity compounds, *e.g.*, fat-soluble vitamins,<sup>7,8</sup> it failed to separate *trans*- $\beta$ -carotene and its cis isomers. Figure 3 shows the chromatograms of an iodine-induced all-*trans*- $\beta$ -carotene solution separated on C18 and C30 columns. Optimizing chromatographic conditions for the separation on the C18 column showed that there was no improvement compared to the conditions optimized for the C30 column. Under the optimized conditions for the C30 column, sixteen peaks were found on the C18 column (Figure 3A), while there were over twenty peaks observed on the C30 column (Figure 3B), demonstrating that the C30 stationary phase was better for separation of for all-*trans*- $\beta$ -carotene and its cis isomers.

## Identification of the Iodine-Induced Isomers of All-*Trans*- $\beta$ -Carotene

Table 2 lists ten peaks from the chromatogram (Figure 3 Panel B) of the iodine-induced all-*trans*- $\beta$ -carotene solution. These peaks were presumed to be the cis isomers of all-*trans*- $\beta$ -carotene based on their similar spectra, shown in Figure 4. When a cis isomerization of *trans*- $\beta$ -carotene occurs at one double-bond, a small absorption peak appears with a maximum absorption wavelength ( $\lambda_{\max}$ ) between 330 nm and 345 nm. This peak is called a cis peak, and the double-bond on which the cis isomerization occurs is called a cis bond. Meanwhile, a slight hypsochromic shift for the  $\lambda_{\max}$  of main peak the cis isomer can be observed, compared to the main peak of all-*trans*- $\beta$ -carotene. The maximum absorption of cis peak ( $A_{\lambda_{\max} \text{ cis peak}}$ ) increased when the cis isomerization occurs close to the molecular center.<sup>1</sup> Table 2 lists the detected  $\lambda_{\max}$  of cis peaks and main peaks. An empirical value put forward by Tsukida *et al.*,<sup>9</sup> Q-ratio, can be used to identify the types of cis isomers. Its value is defined as:

$$Q\text{-ratio} = A_{\lambda_{\max} \text{ cis peak}} / A_{\lambda_{\max} \text{ main peak}}$$

Where  $A_{\lambda_{\max} \text{ cis peak}}$  and  $A_{\lambda_{\max} \text{ main peak}}$  represent the maximum absorption of cis peak and main peak, respectively. Table 2 lists the calculated Q-ratio values of the ten peaks.

Table 2. Identification data for cis isomers of all-*trans*- $\beta$ -carotene.

Peak Number	All- <i>Trans</i> - $\beta$ -Carotene and Its Cis Isomers	Detected $\lambda_{\max}$ of Cis Peak (nm)	Detected $\lambda_{\max}$ of Main Peak (nm)	Q-Ratio Found	Q-Ratio Reported <sup>10</sup>
5	Speculated as false positive cis	331.4	439.8	0.51	—
6	13 or 13'- <i>cis</i> -isomer	329.4	439.1	0.34	0.35
8	Cis not identified	326.6	445.1	0.04	—
9	Cis not identified	341.5	440.7	0.06	—
10	Cis not identified	343.0	437.7	0.09	—
11	Speculated as false positive cis	338.0	451.2	0.79	—
12	15 or 15'- <i>cis</i> -isomer	338.7	443.8	0.41	0.41
13	9,15- <i>cis</i> -isomer	336.2	438.7	0.20	0.20
15	All- <i>trans</i> - $\beta$ -carotene	—	451.4	0.05	0.04
16	9 or 9'- <i>cis</i> -isomer	343.5	446.4	0.10	0.11

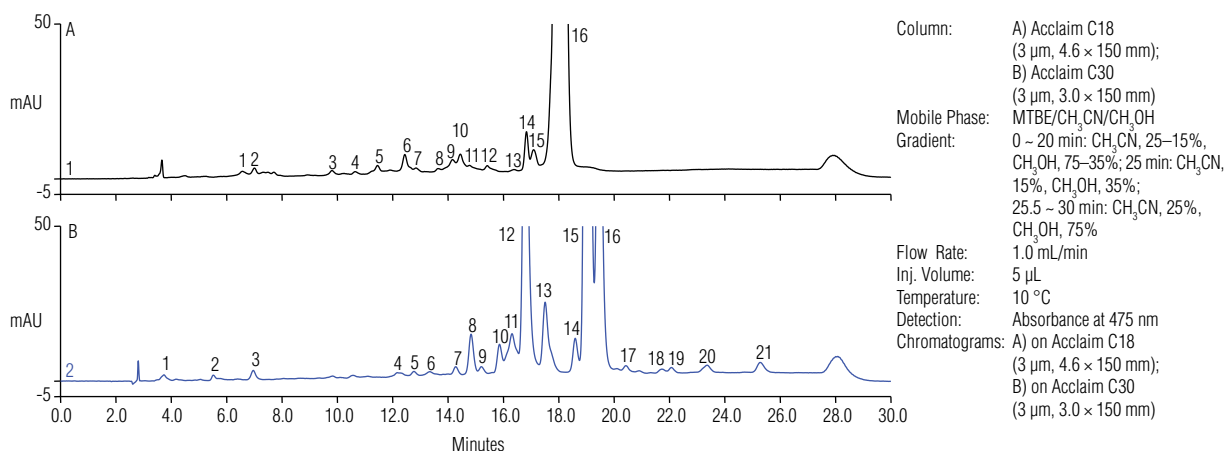


Figure 3. Chromatograms of iodine-induced all-*trans*- $\beta$ -carotene solution using: A) Acclaim C18 (5  $\mu$ m, 4.6  $\times$  150 mm); and B) Acclaim C30 (5  $\mu$ m, 4.6  $\times$  150 mm) columns.

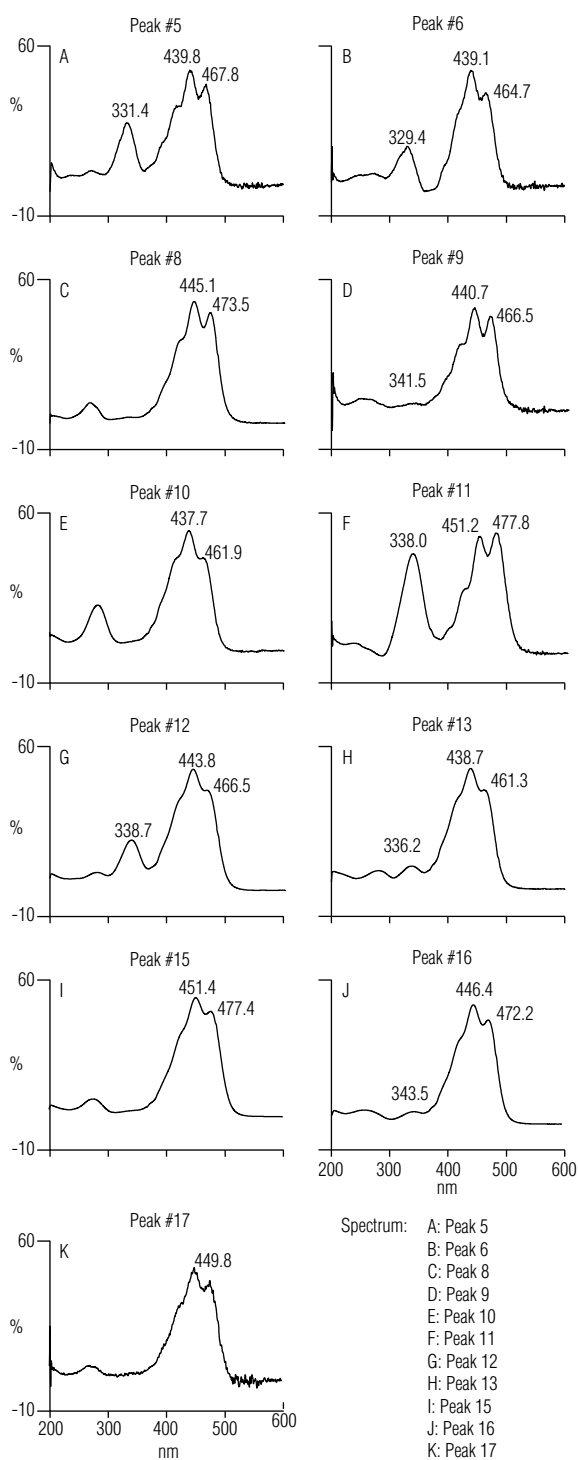


Figure 4. UV absorbance spectra of the compounds separated from iodine-induced all-*trans*  $\beta$ -carotene solution on the Acclaim C30 (5  $\mu$ m, 4.6  $\times$  150 mm) column.

Peak 15 could be positively identified as all-*trans*- $\beta$ -carotene with the standard. Based on the spectral characteristics and reported Q-ratio values,<sup>1</sup> peaks 6, 12, 13, and 16 were tentatively identified as *cis* isomers of all-*trans*- $\beta$ -carotene. Peak 6 was identified as 13 or 13'-*cis*- $\beta$ -carotene, peak 12 was identified as 15 or 15-*cis*- $\beta$ -carotene, peak 13 was identified as 9, 15-*cis*- $\beta$ -carotene, and peak 16 was identified as 9 or 9-*cis*- $\beta$ -carotene. Hypsochromic shifts of more than 6 nm were detected from the main absorption maxima of these *cis*- $\beta$ -carotenes, compared to that of all-*trans*- $\beta$ -carotene.

Although the hypsochromic shift occurred, the structures of peaks 5, 8, and 9 could not be tentatively identified due to the lack of reported Q-ratio values for comparison. The Q-ratio value of peak 10 was similar to that of 9 or 9'-*cis*-isomer (peak 16). However, its detected  $\lambda_{\text{max main peak}}$  (437.7 nm) was significantly different from that of the peak identified as 9 or 9'-*cis*-isomer (446.4 nm) and the reported value (447 nm).<sup>1</sup> Therefore, its identity could not be assigned. The detected  $\lambda_{\text{max main peak}}$  of peak 11 (451.2 nm) was almost the same as that of all-*trans*- $\beta$ -carotene (451.4 nm), as no hypsochromic shift was observed, and no reported Q-ratio value matched its Q-ratio value. Therefore, the structure of peak 11 could not be assigned.

Mass spectrometry detection using an APCI source with SIM scan (537 m/z, span 2) was used for further identification. Peaks 6, 8, 9, 10, 12, 13, 15 and 16 were found, confirming their previous identification using their UV spectra. However, since peaks 5 and 11 were not found, it could be speculated that their identifications based on their UV/vis absorbance spectra were false positives. They did not have Q values to indicate they were *cis*- $\beta$ -carotenes even by UV detection.

#### Faster Separation of the Iodine-Induced Isomers of All-*Trans*- $\beta$ -Carotene

Figure 5 shows a chromatogram of the iodine-induced all-*trans*- $\beta$ -carotene solution using an Acclaim C30 column with a smaller particle size and a narrower diameter (3  $\mu$ m, 3  $\times$  150 mm). The separation was completed with similar resolution in 19 min, and with approximately 56% solvent savings.

Column: Acclaim C30 (3  $\mu$ m, 3.0  $\times$  150 mm)  
 Mobile Phase: MTBE/CH<sub>3</sub>CN/CH<sub>3</sub>OH  
 Gradient: 0-20 min: CH<sub>3</sub>CN, 25-15%; CH<sub>3</sub>OH, 75-35%  
 25 min: CH<sub>3</sub>CN, 15%; CH<sub>3</sub>OH, 35%  
 25.5 - 30 min: CH<sub>3</sub>CN, 25%; CH<sub>3</sub>OH, 75%  
 Flow Rate: 0.7 mL/min  
 Injection Volume: 5  $\mu$ L  
 Detection: Absorbance at 475 nm

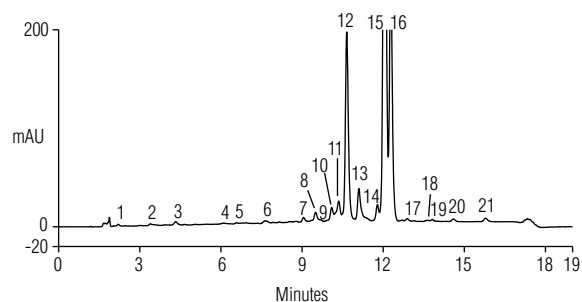


Figure 5. Chromatogram of iodine-induced all-*trans*  $\beta$ -carotene solution using an Acclaim C30 column (3  $\mu$ m, 3  $\times$  150 mm).

## Conclusion

This work demonstrates that the Acclaim C30 column provides an efficient and convenient way to separate  $\beta$ -carotene and its isomers: seven compounds were identified as isomers of  $\beta$ -carotene, and the structures of four isomers were tentatively identified based on their chromatographic behaviors, spectral characters, and mass spectra.

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