Comparing YMC Carotenoid Column with Traditional Reverse Phase Columns in the Analysis of Carotenoids in Nutritional Supplements and Natural Product Extracts

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Best Poster Award Participant

Introduction

Carotenoids are class of natural, hydrophobic, organic molecules found in various fruits, vegetables, and microorganisms. Renown for their antioxidant effect in the body, the natural supplement market focusing on carotenoids is estimated to be a two-billion-dollar industry and expected to grow to five billion dollars per year by 2033. Due to their similar structure, instability, and hydrophobicity, carotenoid content is difficult to analyze by traditional HPLC techniques using C18 media.

In this work, reversed-phase HPLC was used to analyze six specific carotenoid standards, as well as commercially available supplements and extractions from natural products. Method development was performed screening multiple organic phases, gradients, and column media options. Columns screened were YMC Triart C18, penta-fluoro-phenyl, butyl-phenyl, and YMC Carotenoid (C30) phases.

A final method utilizing YMC Carotenoid columns was utilized for the analysis and quantitation of a commercially available supplement, an isomerized β-carotene sample, an extract of commercially available spirulina powder, and an extract of sundried tomatoes. 5µm and 3 µm particle sizes were evaluated for the YMC Carotenoid media, and both columns were sufficient for resolving each carotenoid analyzed as well as resolution of *cis* and *trans* isomers of β-carotene.

HPLC Running Conditions

Column: YMC Carotenoid (C30) 3 µm
Dimensions: 250 x 4.6 mm
Part No.: CT99S03-2546WT
Diluent: 0.1% BHT (w/w) in THF
A: Methanol
Mobile Phase: B: *tert*-Butyl Methyl Ether (MBTE)
C: Water

Gradient: Time (min)	%A	% B	%C
0.0	81	15	4
90	6	90	4
90.1	81	15	4
95	81	15	4

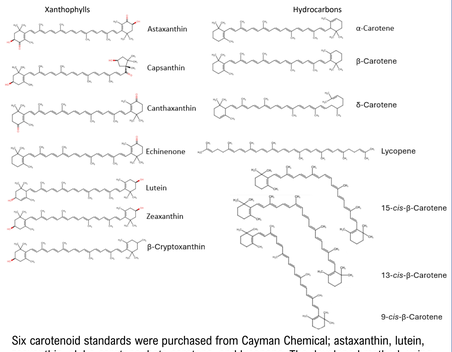
Flow Rate: 1.0 mL/min
Injection Volume: 5 µL
Temperature: 25 °C

LC System: Agilent 1260 Quaternary Pump with DAD

Detection: UV @ 450 nm, 200nm - 500 nm Spectrum

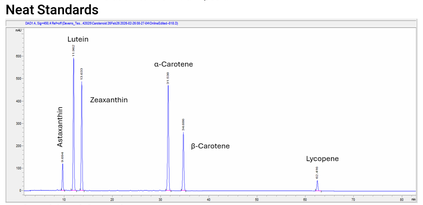
Column Specifications	
Stationary Phase	C30
Bonding	Polymeric
Mode	Reverse Phase
pH Range	2-7.5
Temperature Upper Limit	50°C
Temperature Recommended Range	20-40°C

Carotenoid Compounds of Interest

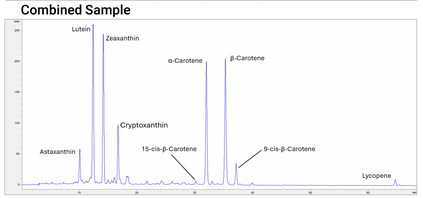


Six carotenoid standards were purchased from Cayman Chemical; astaxanthin, lutein, zeaxanthin, alpha-carotene, beta carotene, and lycopene. The developed method, using a 3 µm particle size YMC Carotenoid (C30) column, adequately resolves the selected standards. Experiments were performed to quantify content in commercially available supplements, natural products, and *cis* isomers of beta-carotene. A combined sample was prepared, combining the standard sample, the spirulina and tomato extract samples, and the iodine catalyzed *cis* isomerization sample. The results show adequate resolution of a wider range of analytes, and highlights resolution between known and unknown isomers in relation to their main peak.

Neat Standards

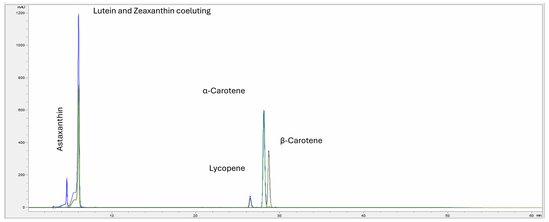


Combined Sample



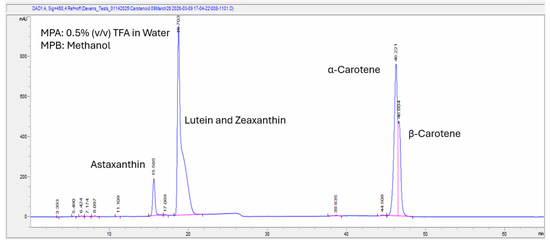
Column Comparison (Best Results Shown)

YMC Triart C18 (250x4.6 mm. 3µm)



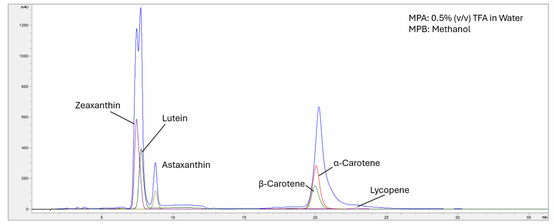
On the C18 column, Lutein and Zeaxanthin co-elute. These two compounds share a structural likeness, differing only in the position of one double bond between two carbons, similar to the difference between alpha and beta carotene. Due to longer retention and partitioning, alpha and beta carotene are able to be resolved. Lycopene elutes earlier than the carotene standards analyzed while it elutes after carotene on the C30 column.

YMC Triart Phenyl (250x4.6 mm, 5µm)



The butyl-phenyl column had poor selectivity between closely eluting isomers (lutein/zeaxanthin and alpha/beta carotene).

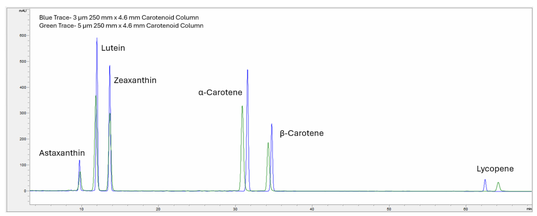
YMC Triart PFP (Pentafluorophenyl) (100x4.6 mm, 3µm)



While resolution of analyzed samples were poor with the penta-fluoro column, a difference in elution order for the xanthophylls was noted. For C18, phenyl, and C30 media, compounds eluted in order of increasing hydrophobicity, with the xanthophyll class well resolved and eluting earlier than the hydrocarbons. While the more hydrophobic hydrocarbons did elute much later than the xanthophylls on the penta-fluoro-phenyl column, the xanthophylls analyzed eluted in order of increasing polarity.

Comparing 3 µm vs. 5 µm YMC Carotenoid Columns

YMC Carotenoid columns with the same dimensions (250x4.6 mm) with different particle size (3 µm and 5 µm) were compared for peak shape, resolution, and sensitivity.

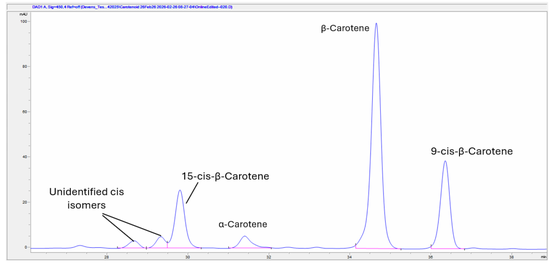


Sample	Compound	Column	Height	Symmetry	Width (50%)	USP Plates	Resolution
100 µg/mL Standard	Astaxanthin	5 µm	73.81	1.01	0.3033	5759	N/A
		3 µm	118.77	0.99	0.1997	13053	N/A
	Lutein	5 µm	369.22	1.07	0.3254	7318	3.84
		3 µm	590.56	1.02	0.2154	17107	6.45
	Zeaxanthin	5 µm	300.83	1.02	0.3358	9168	3.27
		3 µm	485.66	1	0.2178	21713	4.55
	α-Carotene	5 µm	329.34	0.9	0.3379	46217	30.14
		3 µm	468.96	1.04	0.246	91066	45.56
	β-Carotene	5 µm	187.63	0.89	0.3333	58431	5.92
		3 µm	260.61	1.05	0.2463	109897	7.54
	Lycopene	5 µm	34.39	0.88	0.3767	160605	49.69
		3 µm	46.83	0.91	0.2533	336270	65.49

The 3 µm particle size showed a marked improvement over the 5 µm particle size. Peak height showed an increase of 39-61%, USP Plate count increase of 88-137%, USP Resolution improved 27-68%, with more symmetric peaks and a decrease in half-height peak width of 26-34%.

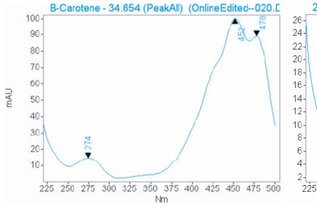
Iodine Catalyzed *cis*-Isomerization of all-*trans* β-Carotene

Carotenes are typically depicted in their all-*trans* form along the conjugated carbon chain. All-*trans* carotenoids are extremely light and heat sensitive, as the stressors can cause a *trans* to *cis* conversion at certain sites, forming *cis* isomers. To demonstrate the resolution capabilities of the YMC Carotenoid column, iodine and UV light were used to force isomerization in an all-*trans* sample of β-carotene.

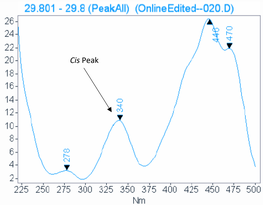


The isomers formed were able to be resolved using the 3 µm particle size YMC Carotenoid column. Notably. The unidentified *cis* isomer closest to the 15-*cis*-β-Carotene peak was unresolved in the 5 µm particle size carotenoid column. *Cis* isomers were identified by collecting a DAD spectrum of the peaks and calculating the peak's Q_{ratio} and comparing it to literature values. This value is the ratio of the height of the "cis-peak" (absorbance at 320–350 nm) to the height of the main absorption peak (typically 430–460 nm).

All *trans* β-Carotene Peak



15-*cis*-β-Carotene Peak

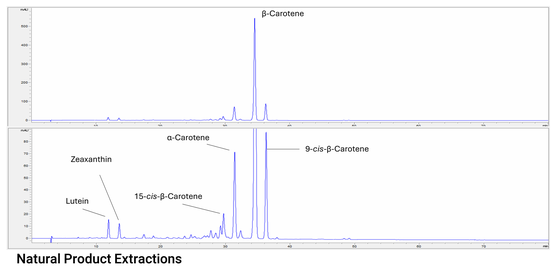


The all-*trans* peak has no local maxima in the 320-350 nm range. Peaks with *cis* bonds present will have a local maxima in the 320-350 nm range. The height of this peak relative to main peak (430-460 nm) is characteristic of different *cis* isomers.

Sample Analysis

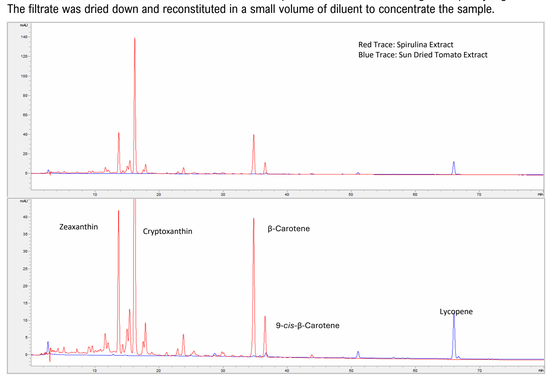
"Eye and Vision Health" Supplement Derived From *Dunaliella salina* Algae

Capsule was emptied into vial containing diluent. Sample diluted to 1,000 µg/mL total carotenoid content based on label claims. Label claims to contain β-carotene, α-carotene, cryptoxanthin, zeaxanthin, and lutein.



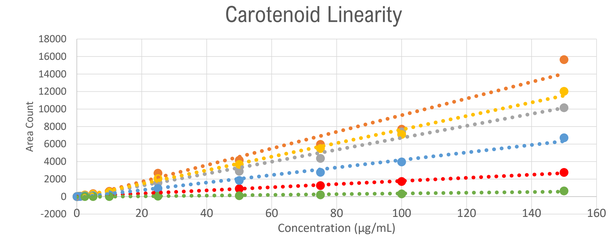
Natural Product Extractions

Freeze-dried spirulina powder and macerated sun-dried tomatoes were separately soaked in diluent and placed in a sonicator set at 40 kHz to assist extraction. The samples were then filtered through a 45 µm syringe filter. The filtrate was dried down and reconstituted in a small volume of diluent to concentrate the sample.



The analytes detected were inline with the expected carotenoids present in each sample according to literature. Extraction efficiency was not explored in this experiment.

Linearity and Quantitation



Linearity samples for each analyte were prepared from standard stock in concentrations from 150 µg/mL to 0.01 µg/mL. Three injections were made at each concentration. The average peak area across the three injections were plotted against sample concentration from LOQ to 150 µg/mL and a linear curve was plotted. The curve was used to quantify the amount of analyte in samples of natural product extracts, as well as over the counter supplements. The amount of analyte in the sample was used with the dilution factor of the sample to calculate the value present in the sample matrix.

Nutritional Supplement

A commercially available nutritional supplement was acquired. The gel capsule was pierced and the contents emptied into a scintillation vial containing diluent. The sample was further diluted until analyte peak areas all fell within the calibration curve. This is reflected in the use of a correction factor when determining the final amount, given in mg of analyte per pill.

Analyte	Slope	Y-Intercept	Sample Peak Area	Correction Factor	Amount (mg/pill)
Lutein	95.036	-206.4	186	108	0.446
Zeaxanthin	68.364	-118.9	146	108	0.418
α-Carotene	77.476	91.2	1227	108	1.583
β-Carotene	42.974	-116.0	7829	108	19.967

The label claims are ambiguous. Label claims 7,500 mcg of Vitamin A as beta-carotene. Additionally, the label claims 108 mg of mixed carotenoids containing beta-carotene, alpha-carotene, cryptoxanthin, zeaxanthin, and lutein, with no claim to the actual quantity of each carotenoid. Quantitative analysis found almost 20 mg of all *trans* beta-carotene and 22.41 mg of quantified carotenoids per pill. There were significant peaks of suspected beta-carotene *cis* isomers which were not quantified.

Spirulina Extract

One gram of dried spirulina powder was soaked in 10 mL of diluent and placed in a sonicator at 40 kHz for two hours to extract carotenoids. The samples were then filtered through a 45 µm syringe filter. The filtrate was dried down and reconstituted in a two mL of diluent. The sample was further diluted until analyte peak areas all fell within the calibration curve. This is reflected in the use of a correction factor when determining the final amount, given in mg of analyte per gram of spirulina.

Analyte	Slope	Y-Intercept	Sample Peak Area	Correction Factor	Amount (mg/g)
Astaxanthin	17.939	-9.6	205	4	0.096
Lutein	95.036	-206.4	245	4	0.038
Zeaxanthin	68.364	-118.9	2955	4	0.360
α-Carotene	77.476	91.2	107	4	0.002
β-Carotene	42.974	-116.0	3313	4	0.638

Literature values give a range of carotenoid content per gram of spirulina. Total carotenoid content is reported between 0.24-4.51 mg/g. Total carotenoids quantified in the sample were 1.13 mg/g, with several significant peaks not quantified (such as suspected cryptoxanthin peak and *cis* isomers of beta-carotene). Beta-carotene content is reported around 1 mg/g with quantified value of 0.638 mg/g. Note that there was no method development performed to optimize the extraction efficiency.

Sun-Dried Tomato Extract

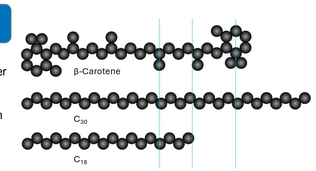
Three grams of sun-dried tomatoes were soaked in 10 mL of diluent and placed in a sonicator at 40 kHz for two hours to extract carotenoids. The samples were then filtered through a 45 µm syringe filter. The filtrate was dried down and reconstituted in a two mL of diluent. The sample was further diluted until analyte peak areas all fell within the calibration curve. This is reflected in the use of a correction factor when determining the final amount, given in mg of analyte per gram of sun-dried tomato.

Analyte	Slope	Y-Intercept	Sample Peak Area	Correction Factor	Amount (mg/g)
Lutein	95.036	-206.4	11	50	0.076
β-Carotene	42.974	-116.0	11	50	0.098
Lycopene	4.088	-49.9	26	50	0.621

Literature gives a range of values for lycopene and total carotenoid content at 0.30-1.07 mg/g and 0.4-1.1 mg/g respectively. Quantitative analysis resulted in 0.621 mg/g of lycopene and 0.795 mg/g of total carotenoids. There is some expected variability based on variety of tomato analyzed, as well as how dry the samples are. These samples were suspected of containing some water content. Note that there was no method development performed to optimize the extraction efficiency.

Results and Discussion

YMC Carotenoid columns are superior to C18 and phenyl columns for carotenoid analysis. The greater number of interaction sites over other media options allows for greater partitioning of large, structurally similar, hydrophobic organic molecules, resulting in sufficient resolution. The resolving power of the Carotenoid column allows quantification of carotenoids in complex sample matrixes where this would not be possible on C18 or phenyl media due to coeluting compounds or structural isomers.



YMC Carotenoid columns are offered in a variety of dimensions and particle sizes. In this work, 5 µm and 3 µm particle sizes were compared. The 5 µm column was shown to sufficiently resolve the main compounds from one another and offers significant advantages over C18 media. In comparison, the 3 µm column in the same dimensions (4.6x250 mm) offers enhanced resolution, symmetry, and sensitivity over the 5 µm. The 3 µm particle offers increased efficiency such that a 3.0x150 mm 3 µm column is comparable to the 4.6x250 mm 5 µm column when flowrate and injection volume is scaled for the reduced diameter.

Conclusion

This work demonstrates that a YMC Carotenoid platform integrating LC/UV with DAD spectra collection provides a comprehensive workflow for carotenoid characterization. LC/UV enabled robust and reproducible quantitation of six targeted carotenoids— astaxanthin, lutein, zeaxanthin, alpha-carotene, beta-carotene, and lycopene. Use of a diode array detector collecting UV spectra data was used to monitor the iodine catalyzed *cis* isomerization of beta-carotene by tracking the formation of a *cis* bond peak ~330 nm and comparing the height of that peak to the height of the main peak ~450 nm.
The capability of the YMC Carotenoid column to sufficiently resolve closely related hydrophobic compounds, as well as hydrophobic compounds across a wide range of hydrophobicity, allows the column to be used to create a method quantifying carotenoids in natural product extracts as well as nutritional supplements.

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