Poster Presentation

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Non-Aqueous Reverse Phase Purification of Carotenes on a Small Particle Preparative Packing

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Introduction

Carotenoids are a class of naturally occurring hydrocarbons consisting mostly of C_{40} molecules varying in the degree of conjugated double bonds. They are ubiquitous in nature and can be found in fungi, bacteria, photosynthetic and non-photosynthetic plant tissue such as algae and seaweed. Some birds, fish, amphibians and reptiles owe their vivid colors to carotenoids. Carotenes and xanthophylls are subclasses of carotenoids; the xanthophylls are oxidized forms of the carotenes and both can be dark red to yellow in color. Figure 1 shows a few of the carotene structures and their absorbance maxima.

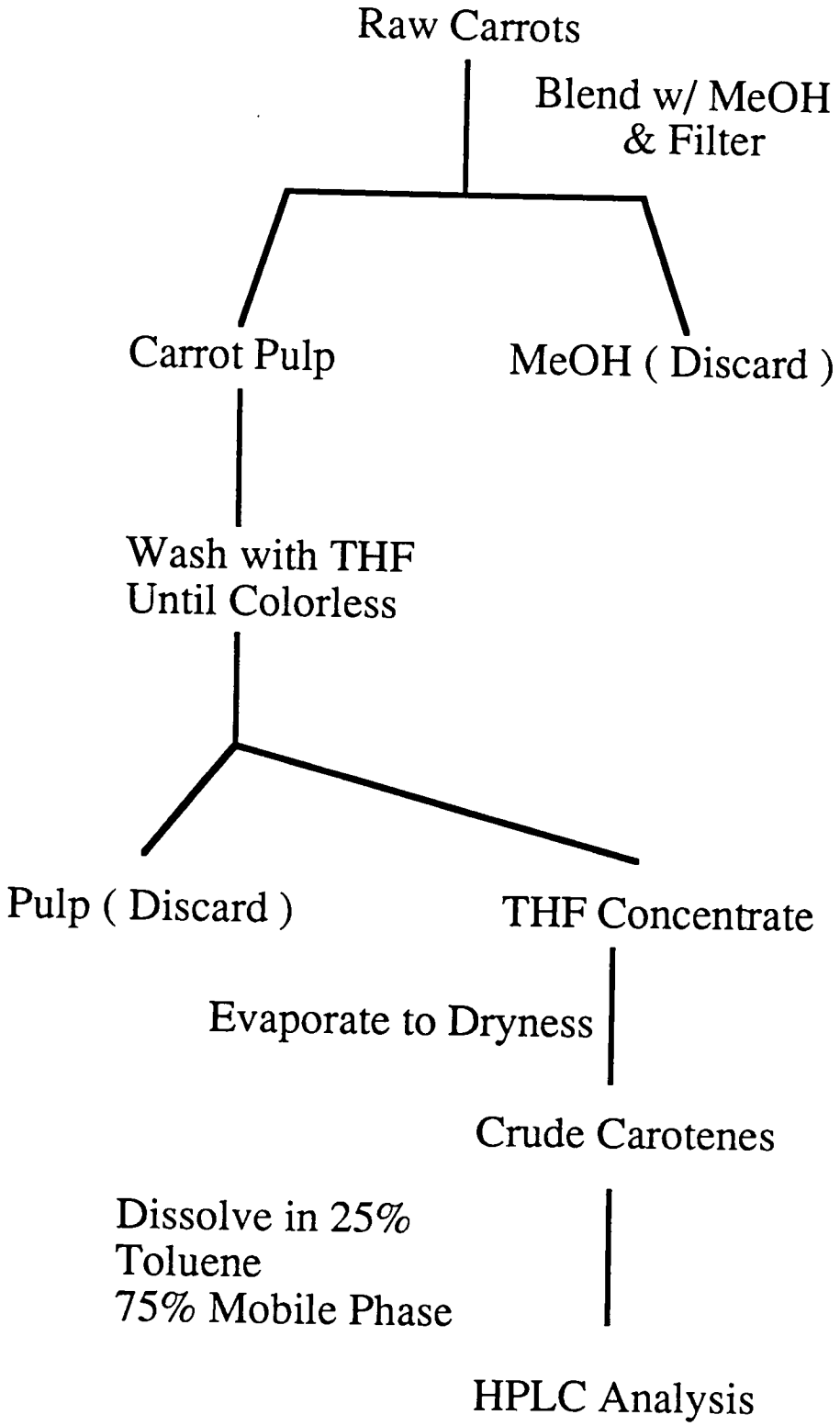
Due to the conjugated nature of these molecules considerable interest in the carotenoids has initiated research endeavors in elucidating the role they play in photosynthetic processes, vitamin A activity in vision and more recently beta-carotene's role as an anticarcinogenic agent[1]. Carotenes are also used as food additives, antioxidants or are available as food supplements. Hence the need for large amounts of pure carotenoids is of paramount importance.

Both analytical and preparative methods for the separation of the classes of carotenoids have been reported in the literature[2-7]. These methods have utilized thin layer chromatography, as well as normal phase and reverse phase column chromatography. Each method has certain advantages and disadvantages and the conditions of elution depends on the sample matrix and the particular carotenoids to be separated or isolated.

Described here is a non-aqueous reverse phase method which separates configurational carotene isomers extracted from a carrot homogenate, and also permits resolution between cis and trans isomers of beta-carotene. In addition this methodology is amenable to scale-up so that larger quantities of purified compounds can be isolated.

Methods

Sample Preparation



Optimization of Analytical Separation:

The separation of the carotenoids was first optimized on a high resolution analytical column (3.9 x 300mm) packed with 4μm Nova-Pak C_{18} material (Figure 2 A,B). Identification of the various carotenoids in the crude mixture was confirmed by comparing relative retention times to known standards and by their characteristic UV/Vis absorbance spectra which were obtained by using an on-line Waters 994 photodiode array detector. Spectra were collected between 200nm and 600nm and were critical in the identification of several of these carotenoids.

Scale Up:

The optimized conditions on the analytical column were transferred to a scaling column (8 x 100mm, cartridge column) packed with a small particle preparative packing, 6μm Prep Nova-Pak HR C_{18} (Figure 2C). A loading study was carried out to determine the maximum amount of sample this column could tolerate under these conditions without compromising resolution of the components of interest. Once established, the flow rate and maximum load on the scaling column can now be used to determine the conditions to be employed for any larger volume column packed with the same particle size and of the same length. The sample load and flow rate are increased proportionally to the cross-section of the packed bed[8] as shown in Equations 1 and 2.

Scaling Equations

$$\text{Mass (prep)} / \text{Mass (scaling)} = r^2 \text{ (prep)} / r^2 \text{ (scaling)} \qquad (1)$$

$$\text{Flow (prep)} / \text{Flow (scaling)} = r^2 \text{ (prep)} / r^2 \text{ (scaling)} \qquad (2)$$

r = column radius

If both column diameter and column length are changing, the flow rate and sample load can be changed proportionally to column volume, as shown in Equations 3 and 4 below:

$$\frac{\text{Flow (prep)}}{\text{Flow (scaling)}} = \frac{\text{Volume (prep)}}{\text{Volume (scaling)}} = \frac{\text{Length (prep)} \cdot r^2 \text{ (prep)}}{\text{Length (scaling)} \cdot r^2 \text{ (scaling)}} \qquad (3)$$

$$\frac{\text{Mass (prep)}}{\text{Mass (scaling)}} = \frac{\text{Length (prep)} \cdot r^2 \text{ (prep)}}{\text{Length (scaling)} \cdot r^2 \text{ (scaling)}} \qquad (4)$$

This results in identical chromatographic performance (retention times, peak widths and resolution) at the preparative scale as that of the scaling column. The flow rate and the maximum sample load on the scaling column were found to be 1.0ml/min (4.0cm/min linear velocity) and 1.2mg (150µl) respectively. Employing equations 1-4 the flow rates and the loads were calculated for three column cartridge configurations, packed with 6µm Prep Nova-Pak HR C_{18} material, which were used in a stackable cartridge holder (Table 1). Chromatography is shown in Figure 3 A,B,C. Fractions were collected during the preparative separation using 25 x 300 cartridge configuration (Fig 3A).

Results and Discussion

Aliquots (50-200μl) of the fractions collected during the preparative separation were analyzed using the high resolution 4μ Nova-Pak column. Samples were injected in the form they were collected i.e. without evaporation or alteration (Figures 4-8). Fraction 8 was identified as phytoene based on its strong absorbance a 286nm[3]. This spectrum is shown in Figure 4. Fractions 1 and 2 have been tentatively identified as cis-zeta-carotene and trans-zeta-carotene based on their UV/vis spectral maxima and retention relative to the other acyclic carotenes[5] (Figures 5 and 6.).

Fraction 3 (Figure 7) was identified as alpha-carotene by its retention and spectral characteristics which were identical to those of a standard. These three fractions together with phytoene represent pure components. Fractions 4,5,6 and 7 contained approximately 80%, 90%, 65% and 30% trans-beta-carotene as the spectral characteristics were very similar to those of a trans-beta-carotene standard. These fractions also exhibited absorptions between 310 and 390nm which had three maxima at 331nm, 347nm and 366nm in addition to absorptions attributable to cis-beta-carotene. A comparison of the three maxima matched those of phytofluene[3,5].

The amount of phytofluene and cis-beta-carotene appears to vary from fractions 4 to 7 suggesting that more than one isomer of each may be present. Several isomers are known to exist for both phytofluene and cis-beta-carotene[5,6]. Therefore it is suggested that fraction 4 contained predominantly trans-beta-carotene with a small amount of phytofluene and cis-beta-carotene. Fraction 5 appears to be most enriched with trans-beta-carotene (>90%) with a small amount of phytofluene. (Figure 8) Fractions 6 and 7 contained decreasing amounts of trans-beta-carotene and what may be other isomers of cis-beta-carotene and phytofluene. Yet the fact that similar spectra are observed from noncontiguous fractions suggest that these other isomers are being resolved from each other however, they coelute with other carotenes found in this mixture.

Conclusion

The difficulty in performing a separation of this type is that all these compounds are closely related and cannot be separated using one simple chromatographic method. The separation must be developed to isolate components of interest. An analytical method for the separation and identification of carotenes in carrot homogenate has been presented. This methodology can be used to scale to larger preparative cartridges and can be used to analyze fractions collected from the preparative separation. The large scale purification of the carotenoids using a 6μm preparative reversed phase packing material permitted the isolation of five different compounds as pure components. Nine different carotenes were tentatively identified by their spectral characteristics and relative retention times. Positive identification of these would require the aid of additional structural analysis such as NMR or mass spectroscopy[5]. Alternative mobile or stationary phases may afford sufficiently different selectivities, thereby resolving compounds which coelute or to separate components not addressed here or from other sources.

References:

1. Burton, G.W. and Ingold, K.U., beta-Carotene: An unusual type of lipid antioxidant. Science 224, 569-575, (1984)

2. Neils, H.J. and De Leenheer, A.P., Isocratic nonaqueous reverse phase liquid chromatography of carotenoids. Analytical Chemistry. 55, 270-275, (1983)

3. Tan, B. and Andersson, A., Analytical and preparative chromatography of tomato paste carotenoids. J. Food Science, 53(3), 954-959, (1988)

4. Bushway, R.J., Separation of carotenoids in fruits and vegetables by high performance liquid chromatography. J. Liquid Chrom., 8(8), 1527-1547, (1985)

5. Davies, B.H., Carotenoids In " Chemistry and Biochemistry of Plant Pigments," T.W. Edwin (Ed.) Vol. 2, pg. 38 Academic Press, Orlando FL. (1976) and references therein

6. Quackenbush F.W., Reverse phase HPLC separation of cis- and trans-carotenoids and its application to beta-carotene in food materials. J Liquid Chrom., 10(4), 643-653, (1987)

7. Scalia, S. and Francis, G.W., Preparative scale reversed-phase HPLC method for simultaneous separation of carotenoids and carotenoid esters. Chromatographia, 28(3,4), 129-132, (1989)

8. Neue, U. Private Communication (1990)

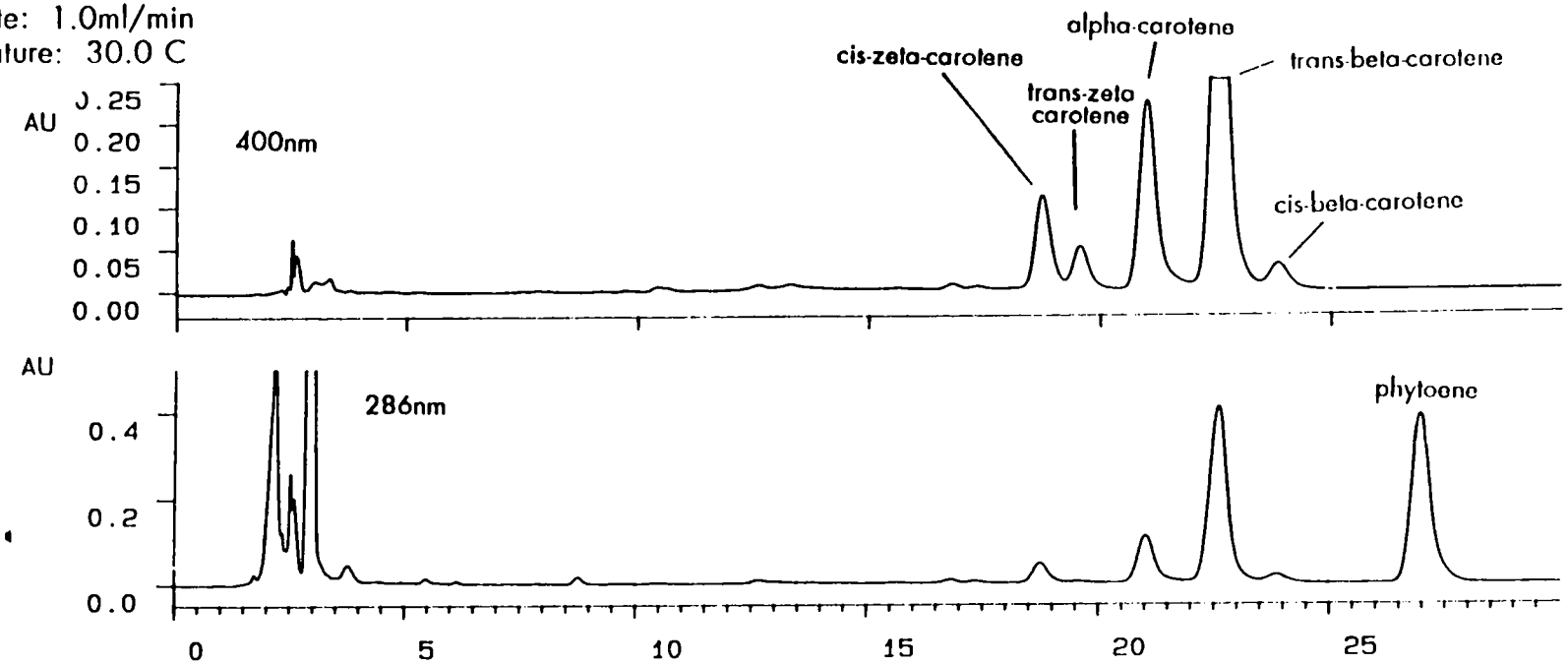
Table 1.

Number of Cartridges	Column Volume	Flow Rate	Sample Load	Run Time
1 x (8 x100mm)*	5.0mls	1.0ml/min	1.2mgs (150µl)	35min
1 x (25 x100mm)	50.0mls	10.0ml/min	12.0mgs (1500µl)	35min
2 x (25 x100mm)	100.0mls	20.0ml/min	24.0mgs (3000µl)	35min
3 x (25 x100mm)	150.0mls	30.0ml/min	36.0mgs (4500µl)	35min

* Scaling cartridge column (8 x 100mm)

Figure 2: Scale-up of Crude Carrot Extract from Analytical to Preparative Particle Sizes

Column: Waters Nova-Pak™ C18, 4µm, 3.9mm x 300mm
Mobile Phase: $CH_3CN/CH_3OH/THF$ 58/35/7
Flow Rate: 1.0ml/min
Temperature: 30.0 C



Column: 8mm x 100mm Radial-Pak™ Cartridge packed with Prep Nova-Pak™ C18, 6µm
Mobile Phase: $CH_3CN/CH_3OH/THF$ 58/35/7
Flow Rate: 1.0ml/min
Temperature: 30.0 C

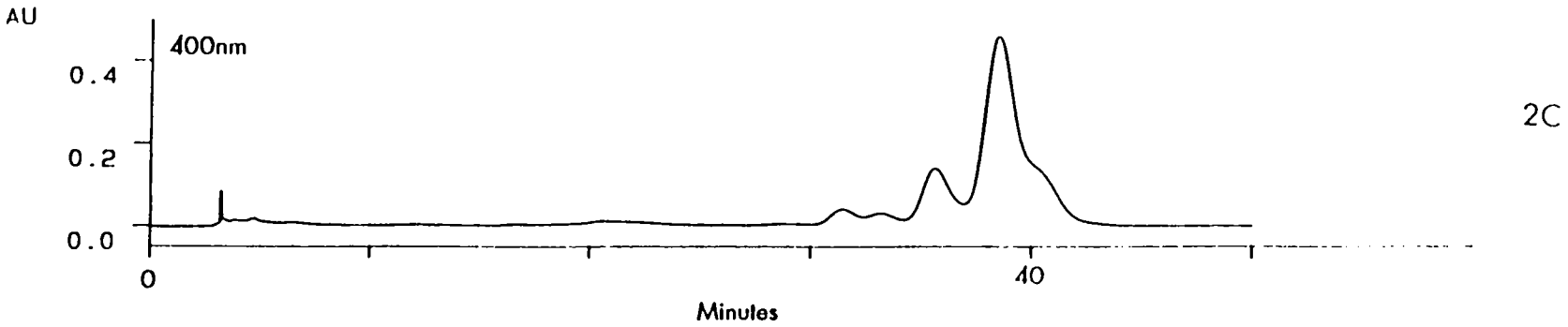
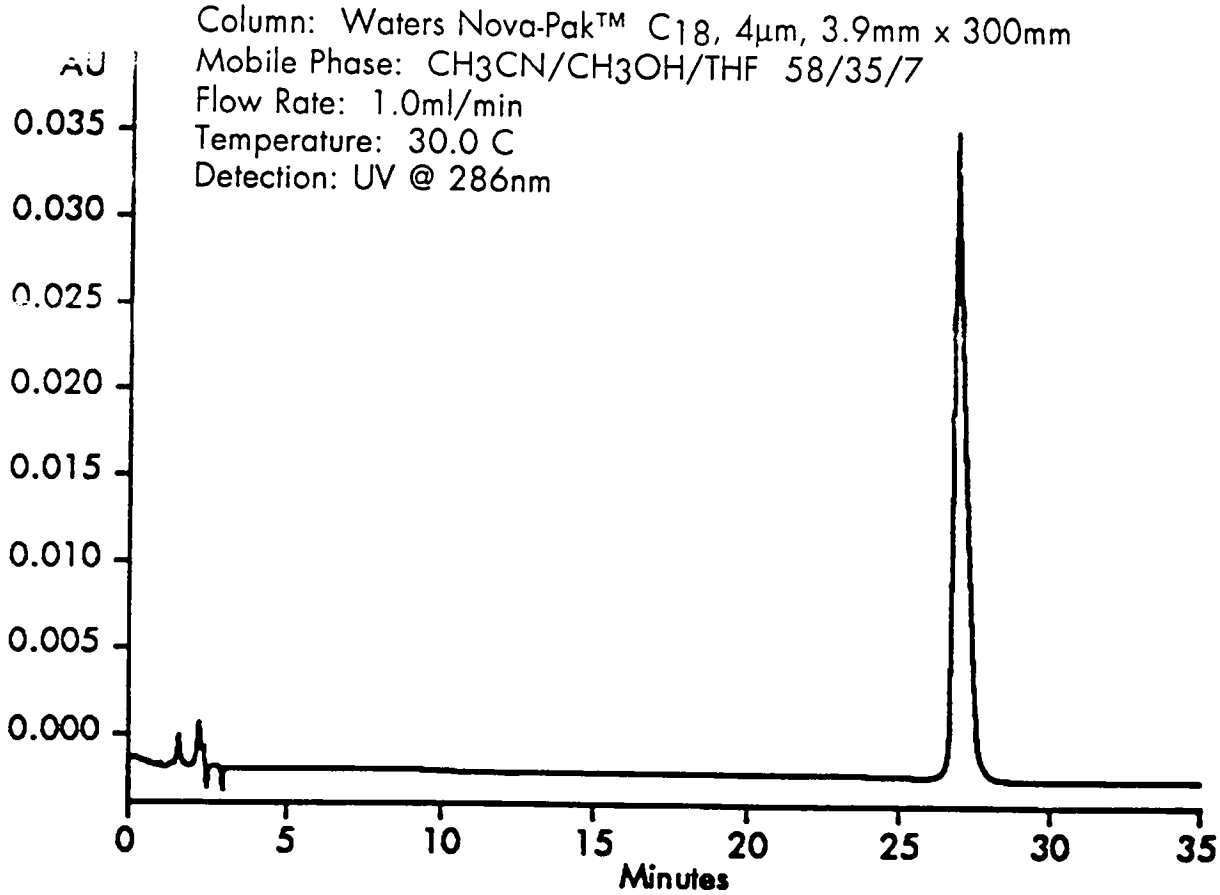
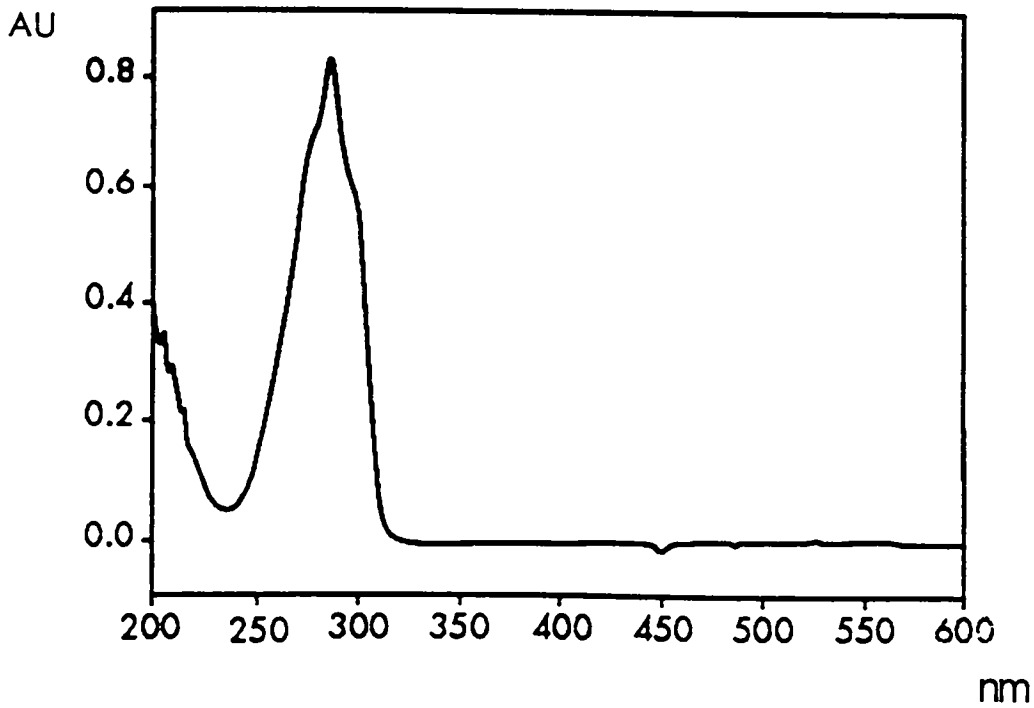


Figure 4: Analysis of Fraction 8 Containing Phytoene.

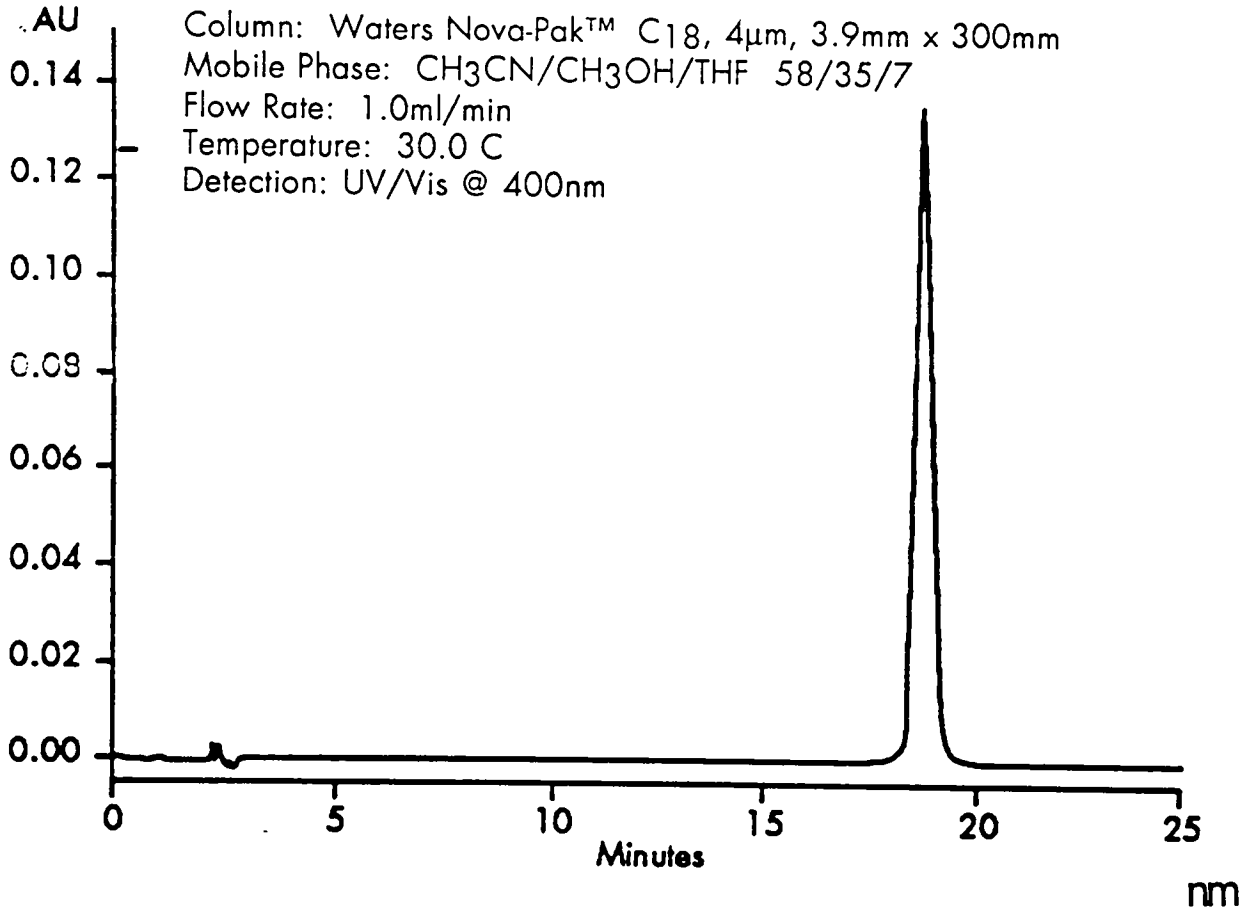


Fraction 8 (46.9-49.2 UV/Vis @ 26.0min)

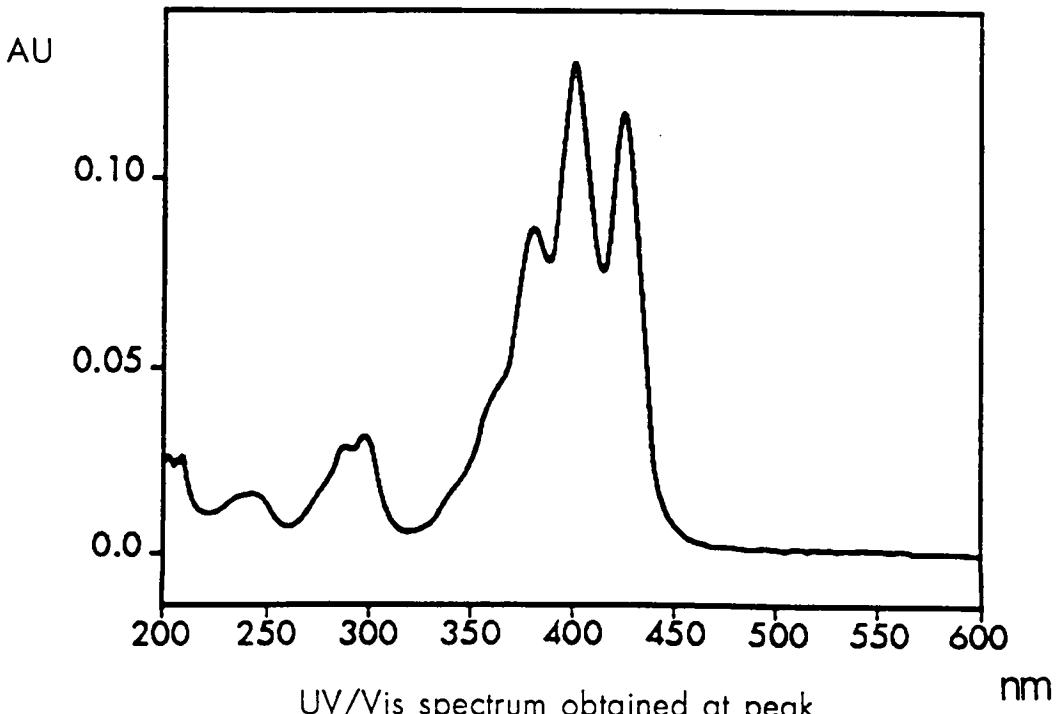


UV/Vis spectrum obtained at peak maxima during analytical separation

Figure 5: Analysis of Fraction 1 Containing cis-Zeta-Carotene.

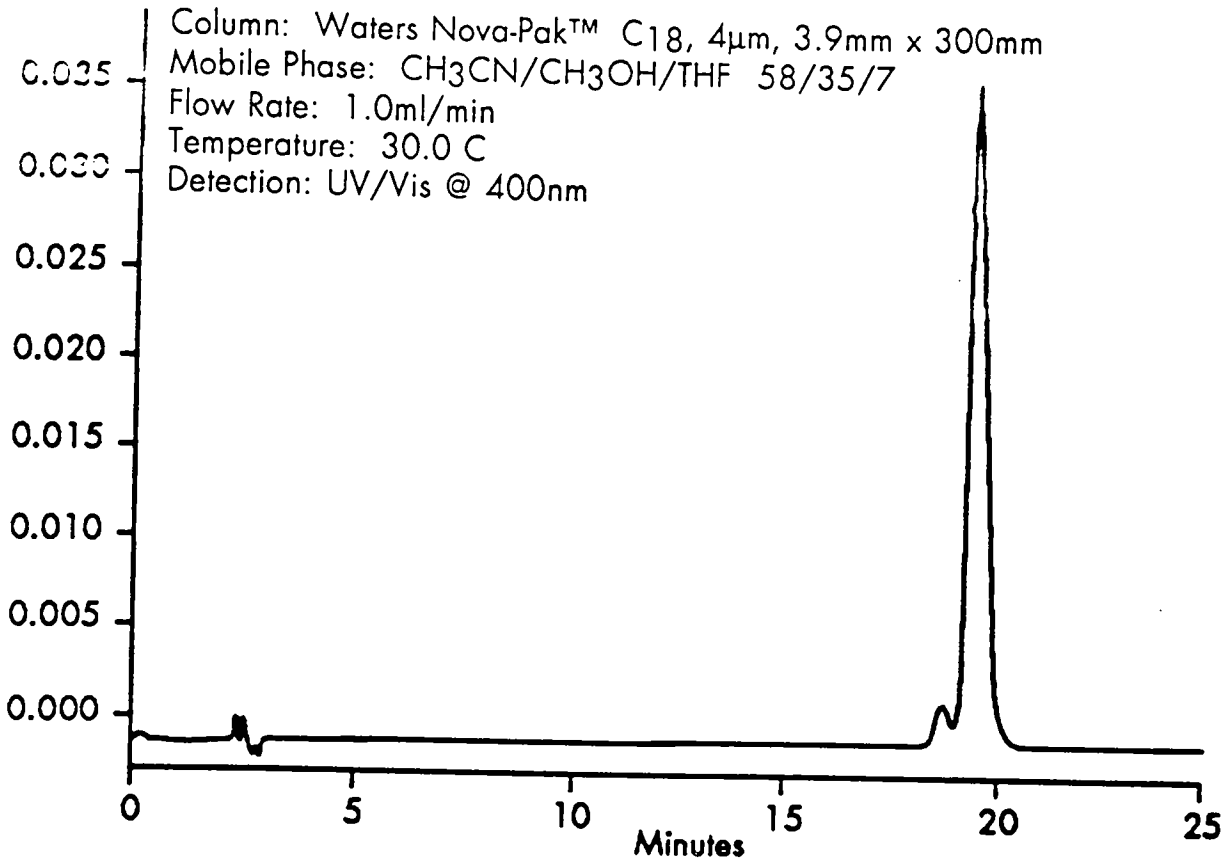


Fraction 1 (32.4-33.8 UV/Vis @ 18.9min)

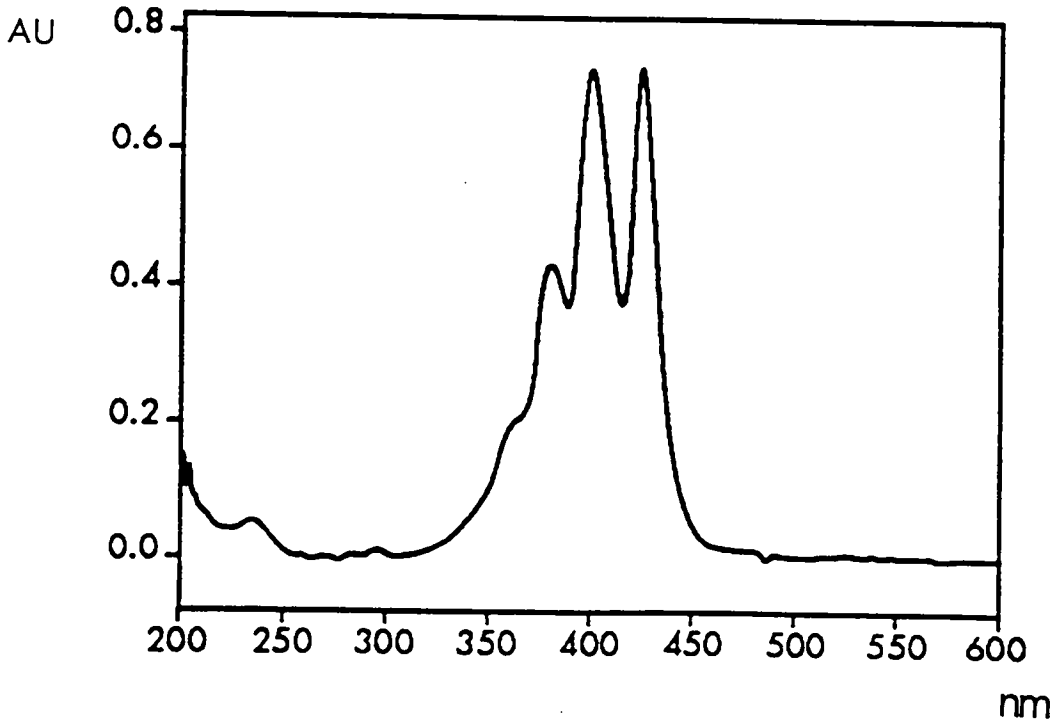


UV/Vis spectrum obtained at peak maxima during analytical separation

Figure 6: Analysis of Fraction 2 Containing trans-Zeta-Carotene.

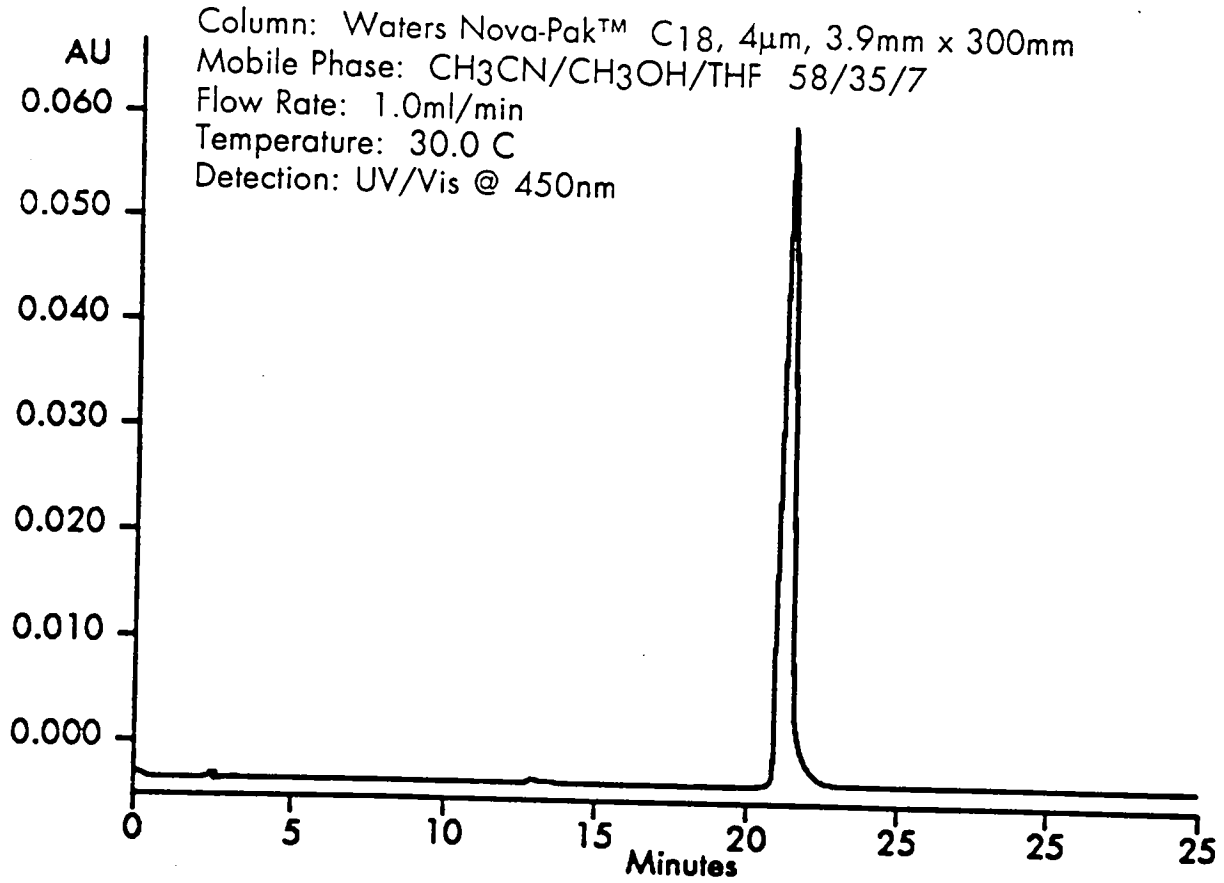


Fraction 2 (34.6-35.4 UV/Vis @ 19.5min)

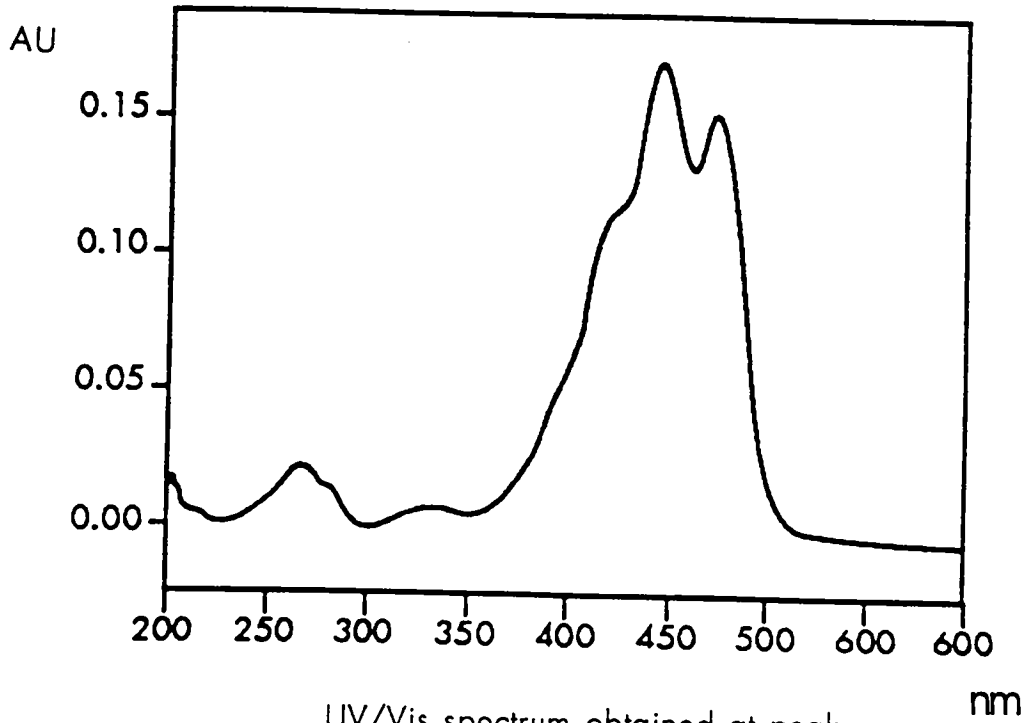


UV/Vis spectrum obtained at peak maxima during analytical separation

Figure 7: Analysis of Fraction 3 Containing Alpha-Carotene.

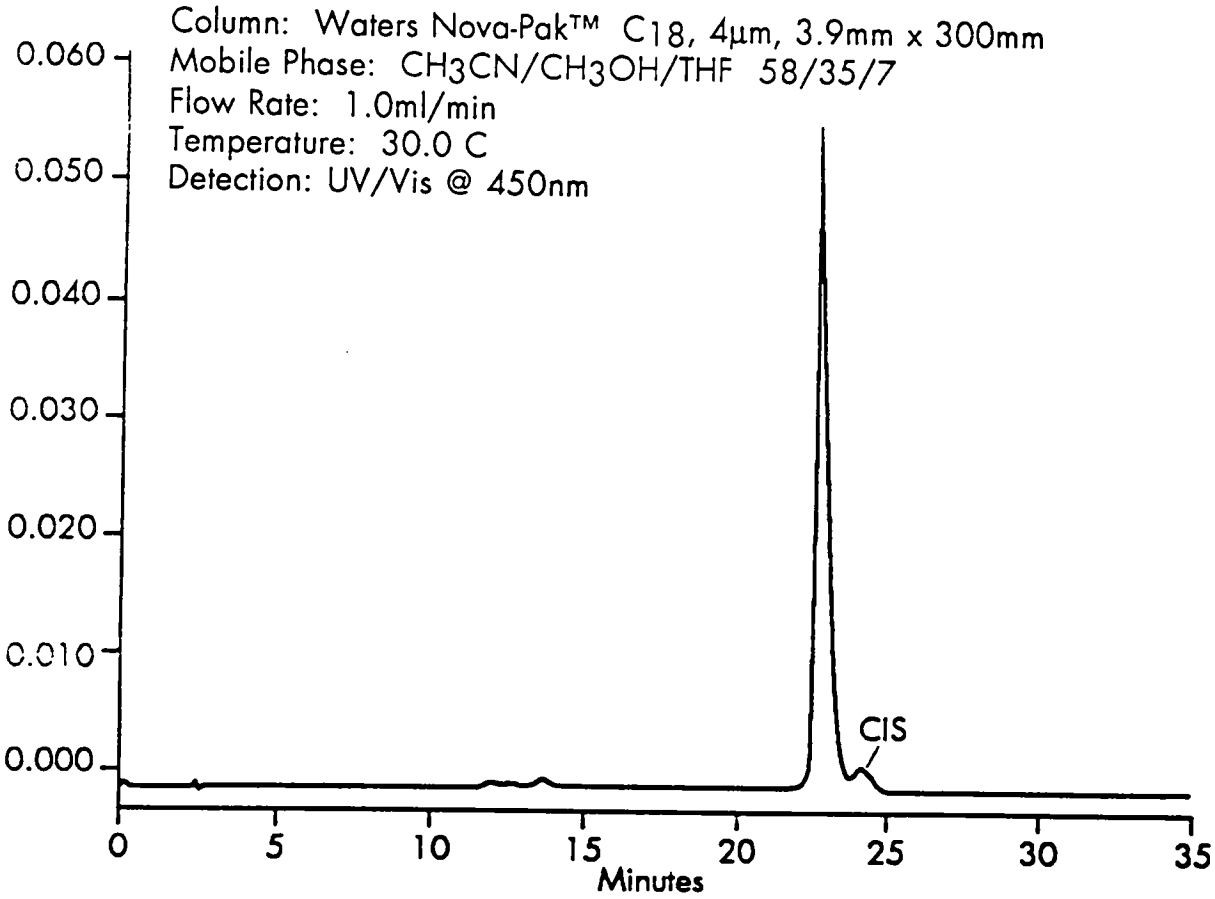


Fraction 3 (36.9-38.4 UV/Vis @ 21.3min)

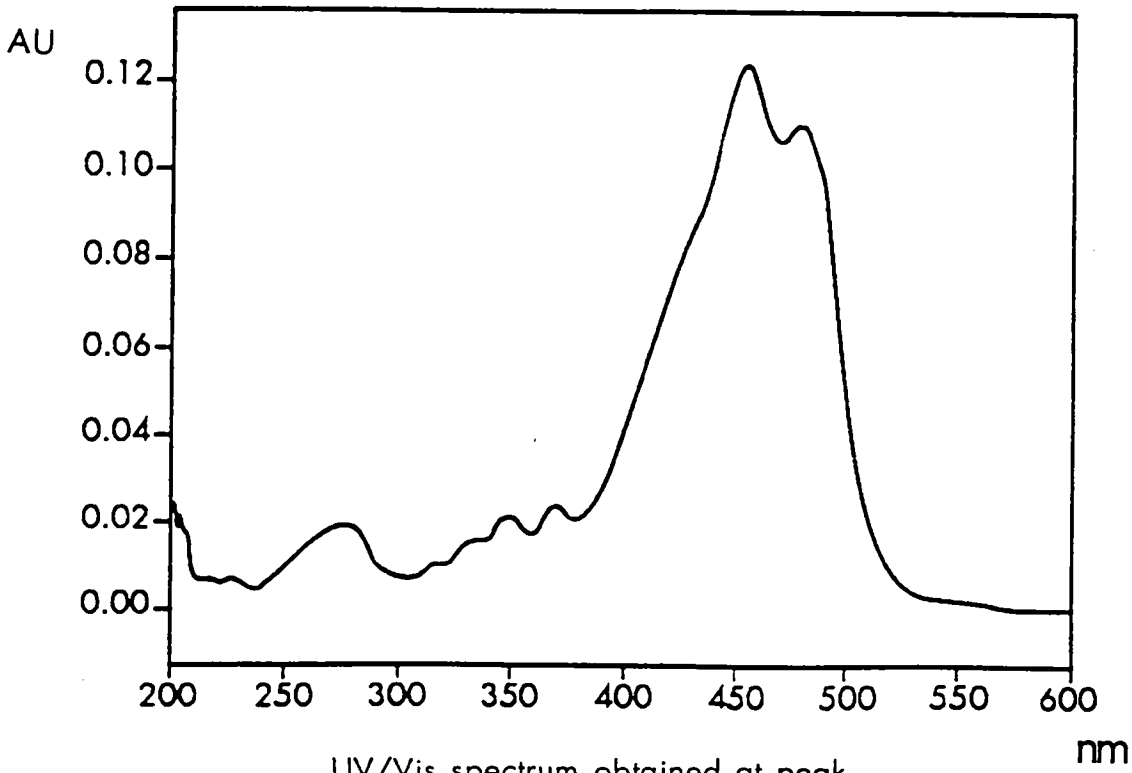


UV/Vis spectrum obtained at peak maxima during analytical separation

Figure 8: Analysis of Fraction 5 Containing trans-Beta-Carotene.



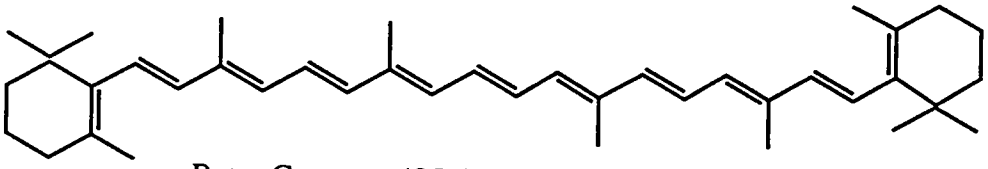
Fraction 5 (40.9-41.7, UV/Vis 22.7min)



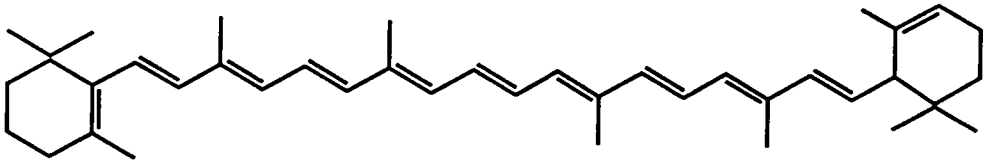
UV/Vis spectrum obtained at peak maxima during analytical separation

Figure 1

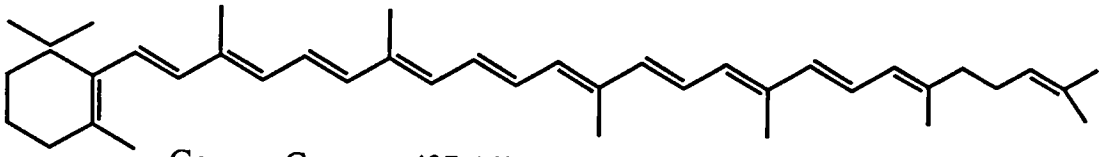
Structures and absorption maxima (nm)* of carotenes[8].



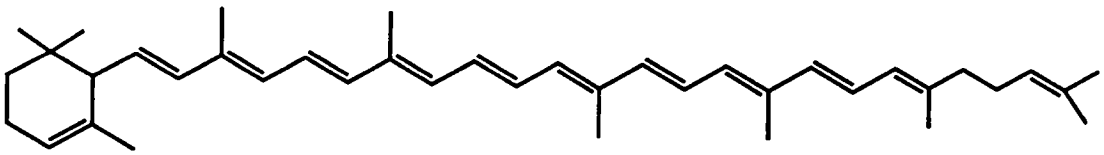
Beta- Carotene 425 450 477



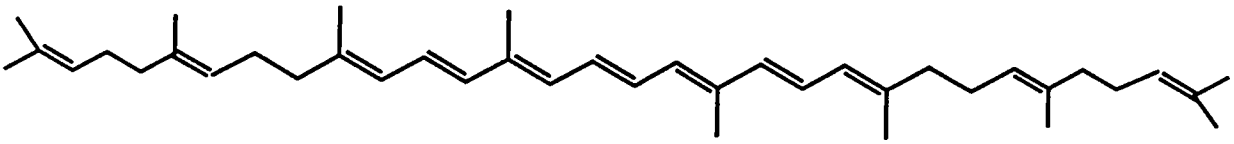
Alpha-Carotene 420 442 472



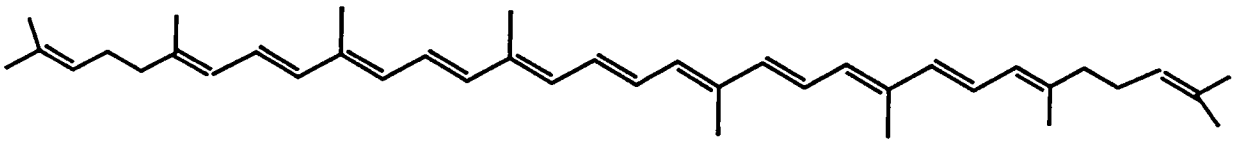
Gamma-Carotene 437 462 492



Delta-Carotene 428 458 490



Zeta-Carotene 380 400 425



Lycopene 448 473 504

* in hexane

Figure 3: Preparative Separation of Crude Carrot Extract using Waters PrepPak® Cartridge Columns

Column: Waters Nova-Pak™ C_{18}, 6µm 25mm x 100, 200 and 300mm Cartridges
Mobile Phase: $CH_3CN/CH_3OH/THF$ 58/35/7
Sample: Crude Carrot Extract
Temperature: 25.0 C
Pump: Waters 600E Multisolvent Delivery System
Detection: UV @ 400nm
Injector: Waters U6K with a 10ml loop