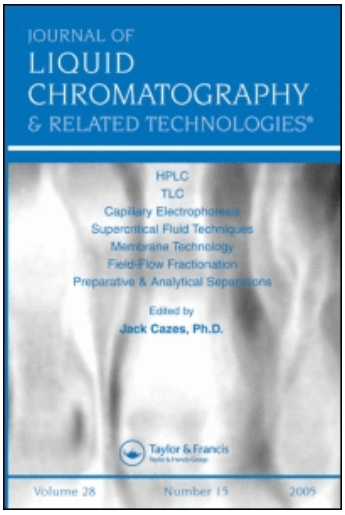

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REVERSED-PHASE HIGH PRESSURE LIQUID CHROMATOGRAPHY SEPARATION OF DEUTERATED COMPOUNDS OF BIOLOGICAL IMPORTANCE FROM THEIR PROTIO ANALOGS

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ABSTRACT

A rapid and a practical HPLC method with UV-VIS detection was developed for the separation and analysis of deuterated carotenoids from their protio analogs. Four different chromatography systems were developed. The results showed that with reversed-phase C_{18} columns it was possible to baseline resolve fully deuterated carotenoids from the nondeuterated analogs. In all instances the deuterated compound eluted ahead of its protio analog indicating that van der Waals forces are operational during the separation process. Specificity, sensitivity, and reproducibility make these methods particularly suitable in plant chemistry for semi-preparative purification processes and methodologies.

INTRODUCTION

Certain photosynthetically active pigments occur in unique structural units of autotrophic plants and are present

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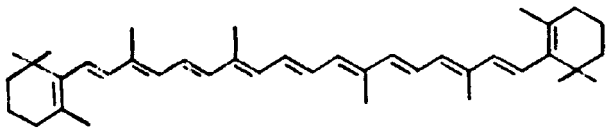
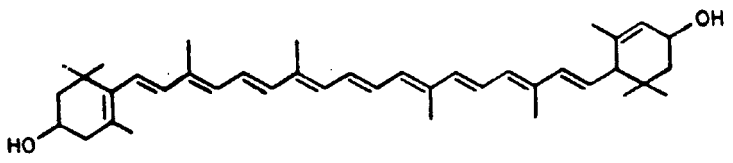
in the colored plastids or chloroplasts of higher plants and various algae. These special chloroplast pigments are divided into three principal groups: the green fat-soluble chlorophylls, the yellow fat-soluble carotenoids, and the red and blue water-soluble phycobilins. The carotenoids are usually subdivided into two groups: the polyene hydrocarbons or carotenes, and their oxy derivatives called xanthophylls. In all truly autotrophic plants the more abundant chlorophyll a conceals the less conspicuous yellow carotenoid pigments.

The separation of chlorophylls and carotenoids was the objective of the classical studies of Tswett using column chromatography. Since then successful modifications have been made (1). Most land plants and most of the green algae yield the same chloroplast pigments. In many instances there is only one carotene, β-carotene. These pigments are separated on a column of powdered sugar using petroleum ether containing 0.5% n-propanol as the mobile phase. Xanthophylls are best resolved on a column of magnesia using petroleum ether containing 25% acetone as the wash. In 1959 it was demonstrated that it was possible to grow organisms under fully deuterated conditions (2). In other words, prior to this period it was believed that the replacement of hydrogen by deuterium was incompatible with life. This discovery opened new fields of isotope chemistry and biology. Many



β - CAROTENE



LUTEIN

FIGURE 1: Chemical structures of β-Carotene and Lutein

species of microorganisms have been cultivated in fully deuterated form, and some species of higher plants and mammals have been partially deuterated. Maximum levels of deuteration have been ascertained and they range from 35% for mammals to 100% for algae, bacteria, fungi, yeasts, and viruses. Replacement of H by D in living organisms is not always detrimental. When green algae (Chlorella vulgaris or Scenedesmus obliquus) are cultured autotrophically in heavy water (99.6% D_2O), the chloroplast pigments contain deuterium instead of hydrogen (3,4). Mixtures of the fully deuterated carotenes and xanthophylls were resolved by chromatography on columns of magnesia with petroleum ether plus acetone as the solvents. Fully deuterated pigments were not separated

from the ordinary hydrogen containing pigments on columns of powdered sugar. On magnesia columns, however, fully deuterated lutein was more strongly absorbed than its protio analog; and fully deuterated β-carotene was strongly held in comparison to ordinary β-carotene (5). Complete separation of the two isotopic forms of β-carotene was observed with the protio compound migrating more rapidly through the column than its deuterio analog. A similar separation but not as complete was observed with the luteins.

There has been a dearth of reports on the HPLC of carotenoids. Separation of carotenoids using HPLC with silica gel as the adsorbent has been studied with selected model carotenoids (e.g. carotenes, diols, cis-trans isomers and diastereoisomers). Fast and efficient separation was obtained on an analytical scale including that of epimers not previously separated. Reversed phase HPLC was used to isolate carotenoid pigments from tomato samples (7) and β-carotene (100% vitamin A-active) was expressed in International units of vitamin A.

MATERIALS AND METHODS

Samples of deuterio β-carotene and protio as well as deuterio lutein were obtained according to procedures reported earlier (1). Protio β-carotene (Lot No. B6A) was purchased from Eastman Kodak Company, Rochester, N.Y. All solvents,

acetonitrile, dichloromethane, tetrahydrofuran, methanol, and acetone were purchased from Burdick and Jackson Labs., Inc., Muskegon, Michigan. Water was prepared by a commercial deionization system (Millipore Corporation). All samples were used without further purification.

All of the RP-HPLC separations were carried out on a Beckman Model 332 liquid chromatograph system equipped with two Model 110A metering pumps under the control of a No. 421 microprocessor. A Hitachi UV-VIS variable wavelength spectrophotometer (Model 155-40) was used as the detector. Samples were introduced by a Model 210 syringe loading injector. Ultrasphere C_{18} ODS, 5μm, 4.6 mm x 250 mm columns with greater than 60,000 theoretical stages/meter (as measured with the standard test mixture) were used. A 0.5μm pre-filter was always inserted before the column and careful attention was directed to the removal of particulate matter from the samples and solvents.

Two milligrams of 'H-β-carotene was dissolved in 5 ml of methylene chloride and 0.25 ml aliquot of this solution was further diluted to 2 ml with additional methylene chloride. A sample of deuterated β-carotene was prepared in a similar manner. In the case of protio lutein, 1.10 mg was dissolved in 2 ml of tetrahydrofuran and then 0.2 ml of this solution was diluted to 2 ml with additional solvent. The solution containing the deuterated isotopic form was prepared in a

similar fashion. All samples were vortexed and syringe filtered. For both β-carotene and lutein, 90 µl of the dilute solution of the protio isotopic form was mixed with 100 ml of dilute solution of the deuterio isotopic form. Mixing unequal volumes of isotopic forms facilitated the identification of peaks. Two microliters of the sample mixture was injected into the chromatograph followed by a 5µl flush with mobile phase. Flow rate was kept at 1 ml/min, and chart speed was maintained at 1 cm/min. β-carotene studies were monitored at 464 nm; lutein was observed at 453 nm. Retention times and capacity factors were calculated after the completion of the run.

RESULTS

β-carotene is the carotenoid most widely found in plants. Deuterated β-carotene and its protio analog were essentially baseline separated on a reversed-phase column using a solvent system of acetonitrile-dichloromethane (70:30 v/v) (Table 1). The run was complete in 19.0 minutes. It was noted that ^{2}H-β-carotene eluted ahead of ^{1}H-β-carotene. This was confirmed by injecting the two isotopic forms separately and matching the retention time values (t_r) with the t_r values recorded for the mixture run. ^{2}H-β-carotene eluted in 15.1 minutes (k' = 4.6) and ^{1}H-β-carotene had a retention time of 17.0 minutes (k'=5.3).

Table 1. Retention Times And Capacity Factor Characteristics For Carotenoids

Carotenoid	Mobil Phase (v/v)	Wavelength of Detection (nm)	Retention Time(min.) ^2H	Retention Time(min.) ^1H	Capacity Factor(k') ^2H	Capacity Factor(k') ^1H
Beta Carotene	Acetonitrile: Dichloromethane 70:30	464	15.1	17.0	4.6	5.3
Beta Carotene	Acetonitrile Tetrahydrofuran 70:30	454	9.7	10.7	2.6	2.9
Lutein	Methanol:Water 95:5	453	11.6	12.6	2.8	3.3
Lutein	Acetone:Water 75:25	453	17.4	19.8	4.6	5.4

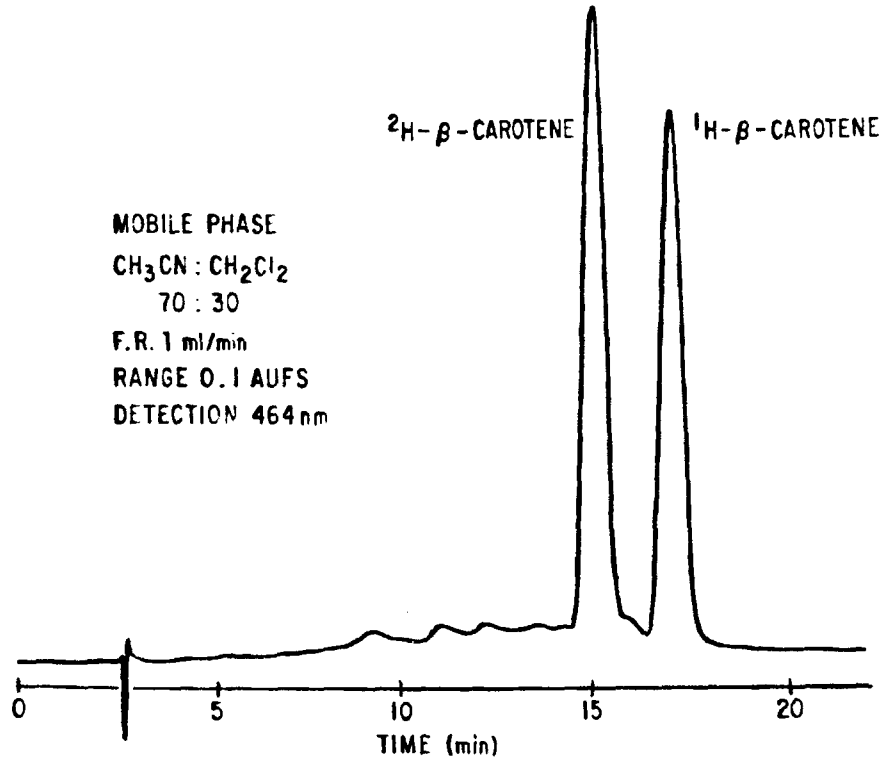


FIGURE 2: Reversed-Phase (C_{18}) - HPLC separation of ^2H-β-Carotene from its protio analog.

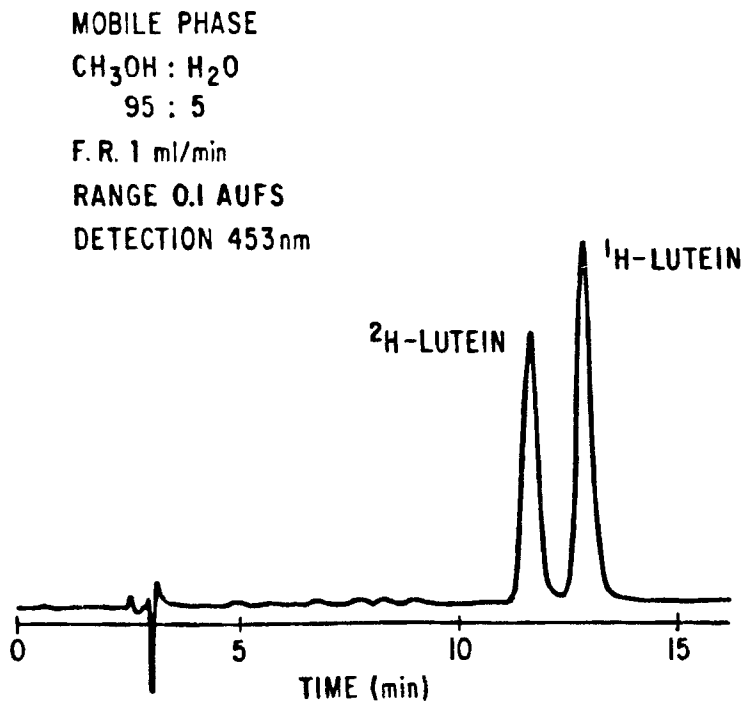


FIGURE 3: Baseline separation of ^{2}H-Lutein from ^{1}H-Lutein on a C_{18} reversed phase column.

A second solvent system which also provided satisfactory separations consisted of acetonitrile: tetrahyrofuran (70:30 v/v). This system gave a more rapid separation that the previous one. ^{2}H-β-carotene eluted in 9.7 minutes (k'=2.6) and ^{1}H-β-carotene had a retention time of 10.7 minutes (k' = 2.9).

Lutein possesses two hydroxy groups and is relatively more polar than β-carotene. Methanol: water (95:5 v/v) gave baseline separation for a mixture of the two isotopic forms

of lutein. Separation was effected in 14.0 minutes with ^{2}H-lutein eluting ahead of ^{1}H-lutein with a t_r value of 11.6 minutes (k'=2.8). The protio analog had a retention time of 12.8 minutes (k'=3.3). Each isotopic form of lutein was run separately and the retention time values were compared with those obtained for the mixture. Identical values were recorded. Acetone: water (75:25 v/v) gave suitable separation but the peaks were broader and the run took 22.0 minutes; longer than for the previous system. In this system ^{2}H-lutein eluted in 19.8 minutes (k'=5.4).

Both protio β-carotene and protio lutein can be separated from their respective deuterio forms by RP-HPLC. In both instances, the deuterio compound eluted faster than the protio analog.

DISCUSSION

In chromatographic separations of carotenoids (β-carotene and lutein), the deuterated compound eluted ahead of its protio analogs. At the outset if appears that such chromatographic behaviour is independent of the size of the molecule. Relatively greater solubility of the deuterio isotope in the mobile phase is apparently not a factor. If solubility were a factor then in some solvent system it would be expected that the deuterio compound would elute more slowly. Regardless of the composition of the solvent

system, the deuterio compound always eluted first. The elution of the ^{2}H-isotopic form before its protio analog suggests then that van der Waals force are operational. Van der Waals forces used in this context also encompass the Keesom, Debye, and London attraction forces. A C-H bond has a higher oscillation frequency (3300 cm^{-1}) than the C-D bond (2333.8 cm^{-1}). With oscillation frequency an electromagnetic field is created and the electrons in the C-H bond are subject to this field. In turn, this electromagnetic field for a C-H bond creates a large induced field of opposite charge in other molecules around it. The C-H bond induces greater forces of attraction between itself and the stationary phase. The C-D bond, on the other hand, has a lower oscillation frequency, a less electromagnetic field is created, and the forces of attraction that develop between the C-D bond and the stationary phase are weaker. Therefore, the C-D bond with a lower van der Waals force of attraction elutes ahead of its protio analog.

Further explanation is forthcoming on the basis of the chemical potential (μ) of a component which is a measure of its escaping tendency from one phase to another. It is an intensive property, i.e., it is independent of the number of moles of the components. The chemical potential of a component is identical in all phases of a heterogeneous system when the phases are in equilibrium at a fixed temperature

and pressure. Each chromatographic theoretical plate is, in essence, an equilibrium between the mobile phase and the stationary phase. The escaping tendency of the solute is the same in all parts of the system. A quantitative measure of the escaping tendency for constituents of a solution undergoing physical and/or chemical transformations is the partial molar free energy, $(\frac{\Delta F}{\Delta n})_{T,P}$.

$(\Delta F°_{298})_{liquid}$ for D_2O is -243.5 kJ/mole and for H_2O it is -237.2 kJ/mole. The negative sign, by convention, signifies that the process is spontaneous. On the basis of higher negative value for D_2O it may be assumed that the escaping tendency for deuterio compound from the mobile phase to the stationary phase and vice versa is greater that its protio analog. It is then quite understandable that the deuterio solute possesses greater spontaneity and escaping tendencies than its protio analog. Consequently, the equilibrium processes for the deuterio compound are established faster and it moves ahead quicker than the compound of ordinary isotopic composition.

Reversed-phase high pressure liquid chromatography has been shown to be a versatile tool for separating isotopically analogous compounds. In this study the compounds were identical in all respects except the C-H and the C-D bond which differ in frequency of vibration. Successful separation may be attributable to the very large number of theoretical

plates (plate count > 60,000 per meter) attainable with commercially available chromatography columns. It appears that the larger the aliphatic C-H component in the compound, the easier will separation be effected. With the increased popularity of HPLC and the availability of more efficient columns, isolation, purification, and quantification studies can now be performed.

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