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Oxidation of Zeaxanthin and characterization of 3'-Alkyl Lutein ethers

Jie Chi

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FLORIDA INTERNATIONAL UNIVERSITY

Miami, Florida

OXIDATION OF ZEAXANTHIN AND
CHARACTERIZATION OF 3'-ALKYL LUTEIN ETHERS

A thesis submitted in partial fulfillment of the

requirements for the degree of

MASTER OF SCIENCE

in

CHEMISTRY

by

Jie Chi

2001

To: Dean Arthur W. Herriott
College of Arts and Sciences

This thesis, written by Jie Chi, and entitled Oxidation of Zeaxanthin and Characterization of 3'-Alkyl Lutein Ethers, having been approved in respect to style and intellectual content, is referred to you for judgment.

We have read this thesis and recommend that it be approved.

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John T. Landrum, Major Professor

Date of Defense: April 6, 2001

The thesis of Jie Chi is approved.

Dean Arthur W. Herriott
College of Arts and Sciences

Interim Dean Samuel S. Shapiro
Division of Graduate Studies

Florida International University, 2001

DEDICATION

I dedicate this thesis to my parents, my husband, and my daughter. Without their patience, understanding, encouragement, and unconditional love, this work would not have been finished.

ACKNOWLEDGMENTS

It is a pleasure to acknowledge the help given to me by so many people during my study at Florida International University. I wish to express my sincerest gratitude to my major professor, Dr. John T. Landrum, for all of his the patience, concern, encouragement, and guidance. I would like to thank my other committee members, Dr. Richard A. Bone, Dr. J. Martin E. Quirke, and Dr. Yong Cai, for all their advice, encouragement, and critical review of the final thesis. Dr. Bone has given me help in every detail during my residence in his lab. Dr. Quirke and Dr. Cai have given me many suggestions. I am thankful to Dr. Deng, Dr. Lopez, Dr. O'Shea, Dr. Wnuk, and other faculty and staff members in the Chemistry Department for their kind help and advice. Special thanks are due to Mr. Alberto Sabucedo who has given me so much help in obtaining mass spectra. I would like to thank Christian Herrero, Yin Chen, and all the co-workers in our labs for their help and friendship.

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ABSTRACT OF THE THESIS

OXIDATION OF ZEAXANTHIN AND

CHARACTERIZATION OF 3'-ALKYL LUTEIN ETHERS

by

Jie Chi

Florida International University, 2001

Miami, Florida

Professor John T. Landrum, Major Professor

One purpose of this study was to understand the oxidation metabolites of zeaxanthin, another was to prepare, purify, and characterize a series of 3'-alkyl lutein ethers for use as internal standards.

Studies have proven that lutein and zeaxanthin are two of the principal carotenoids in human serum and the only carotenoids found in the retina, but their metabolism and transport in the human body are still only poorly understood. *In vitro* oxidation of zeaxanthin with MnO₂ produced three components. They were characterized by HPLC, UV/Vis, MS, NMR and identified as all-trans rhodoxanthin and its cis-isomers.

3'-alkyl lutein ethers have been utilized as internal standards for carotenoid analysis in human serum and macular tissue. Nine 3'-alkyl lutein ethers were prepared by reaction of lutein with the corresponding acidified alcohols. The purified products were characterized by HPLC, UV/Vis, MS for the first time, and the resulting lutein ethers showed similar properties to lutein with increasing retention times as the alkyl chain lengthened. This provides us alternatives in selecting internal standards.

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Chapter I Introduction

Carotenoids are natural occurring pigments and widely found in colorful plants, algae and bacteria^{1, 2}. Animals including human beings can not synthesize carotenoids by themselves, they must consume and absorb carotenoids from their diet.

1.1 Occurrence

More than 600 hundred carotenoids have been found in nature¹. The most abundant naturally occurring carotenoids, in particular those of higher plants, are the C₄₀ tetraterpenes^{1,2}. Carotenoids which have a C₄₀ structure but only contain hydrogen and carbon are called carotenes. Xanthophylls are oxygen-containing C₄₀ carotenoids. Other carotenoids may have structures with either more or fewer than 40 carbon atoms³. The biosynthesis of carotenoids occurs by the well-established terpenoid pathway⁴. C₄₀ carotenoids are built up from eight isoprene units (C₅H₁₀) through “head to tail” condensation¹. In Fig.1.1, lycopene (ψ,ψ -carotene) is used as an example to show this.

Fig.1.1 shows some carotenoids commonly found in nature. Lycopene (ψ,ψ -carotene) (in tomato) is an example of an acyclic carotene, β -carotene (in carrot) with two β - rings is an example of an alicyclic carotene. The other five carotenoids are alicyclic xanthophylls. Lutein (one β - and one ϵ - ring) and zeaxanthin (two β - rings) are two kinds of carotenoids commonly found in algae and plants. Astaxanthin and canthaxanthin are found in the skin and flesh of some fish (salmon, shrimp). Among the carotenoids which possess a retro polyene chromophore, rhodoxanthin is a well-known example and is found in yew berries (*Taxus baccata*)⁵, bronze winter needles of *Cryptomeria*⁶,

Equisetum Arvense sporophytes⁷, fungus *Epicoccum nigrum* (Deuteromycetes)⁸, some bird feathers⁹, and in fish¹⁰.

Fig.1.1. Some carotenoids commonly found in nature.

Carotenoids can be obtained by isolation from their natural sources. Synthesis (including partial synthesis²-in which a chemical transformation is employed with the carotenoid skeleton unchanged) is another way to obtain a specific carotenoid compound. Nearly 200 naturally occurring carotenoids have now been synthesized, and some of them are commercially available. Recently, scientists have developed a method for preparing samples of carotenoids using genetic engineering techniques¹¹. They isolate carotenoid genes using a DNA-shuffling technique then introduce these genes into bacterial host cells or plants to obtain a variety of carotenoids and even to create some new ones.

1.2 Functions

Carotenoids are widely used in the food, cosmetic and pharmaceutical industries. According to the statistics data from SRI Consulting (Menlo Park, CA) ¹¹, the 1999 world market for carotenoids was estimated to be \$750-800 million per year. Another report (Chemical Week, September 6, 2000, v.162, 33, 25) mentions that the worldwide market for carotenoids will grow 2.9%/year, to \$935 million, by the year 2005.

Pro-vitamin A

Carotenoids are the main source of vitamin A in human and animal nutrition. The most important metabolite of carotenoids is vitamin A. Vitamin A is not only essential to the eye, it has important systematic functions including maintaining growth and reproductive efficiency. The mechanism of the conversion of β -carotene to retinol via central cleavage has been widely discussed^{12,13}. The discovery of the relationship between carotenoids and vitamin A has greatly stimulated further research in the area.

K.Schiedt et al.^{14,15} concluded that canthaxanthin, astaxanthin and zeaxanthin are pro-vitamin A compounds which are converted into retinol in rainbow trout, salmon and chicken. T.Matsuno¹⁶ discovered xanthophylls (such as canthaxanthin, astaxanthin, zeaxanthin, lutein and tunaxanthin) are precursors of retinoids through a metabolic pathway from 3-dehydroretinol to retinol in feeding experiments with fish and rats.

Fig.1.2. β -carotene and vitamin A.

Color Additives

Carotenoids are widely used as color additives in the food and cosmetic industry. As the additives to food and animal feed, they have functions both for coloration and as a vitamin A supplement.

Photosynthesis and Photoprotection

In the photosynthetic systems of plants, algae and phototrophic bacteria, carotenoids protect against photosensitized oxidation. In non-photosynthetic organisms like some

bacteria, carotenoids can also diminish photooxidative damage caused by singlet oxygen.¹⁷

Protection Against Disease

Many clinical studies and animal tests show carotenoids can efficiently prevent or reduce the risk of certain kinds of diseases. Clinical evidence indicates that increased dietary intake of foods that are high in carotenoids, like lutein, may help prevent colon cancer¹⁸, lung cancer¹⁹, prostate cancer²⁰, and breast cancer²¹. Other clinical studies^{22,23} suggest that dietary or supplementary intake of lutein may reduce the risk of two common sight robbers—cataracts and age-related macular degeneration (AMD). The latter is the leading cause of acquired blindness in persons over the age of 65 in the U.S. Experts believe that carotenoids (like lutein) can screen out harmful blue ultraviolet light and act as an antioxidant²³.

1.3 Methodology

Qualitative Analysis

In qualitative analysis, UV/Visible spectrophotometry (UV/Vis), high-performance liquid chromatography (HPLC), mass spectrometry (MS), infrared spectrometry (IR), Nuclear Magnetic Resonance Spectrometry (NMR) are used. In some cases HPLC coupled with photodiode-array detector (PDA) is used to obtain on-line information. Circular dichroism (CD) is also informative.

Carotenoids have the fundamental property that they absorb light in UV or visible wavelength range and undergo a $\pi \rightarrow \pi^*$ transition in the conjugated double-bond system. The longer the conjugated double-bond chain [the larger the number of

conjugated double bonds (c.d.b.)), the less the energy needed for the transition, and the longer the wavelength at the maximum absorption (λ_{max}). The ratio of the peak heights III/II (Fig.1.3) is often calculated to present the spectral fine structure²⁴.

Fig.1.3. The method of calculating %III/II.

According to the UV/Vis spectra (in ethanol) in the literature²⁴, lutein, which has 10 c.d.b.s and one β - ring and one ϵ - ring like β , ϵ -carotenes, has a λ_{max} at 445 nm and %III/II value of 60; zeaxanthin, which has 11 c.d.b.s and two β - rings like β -carotenes, has a λ_{max} at 450 nm and %III/II value of 25; rhodoxanthin, which is a conjugated keto compound and has 14 c.d.b.s (including two carbonyl groups), has a λ_{max} at 496 nm and exhibits only one broad peak without fine structure (%III/II=0). ψ,ψ -carotenes (lycopene)

has a %III/II value of 65. In some other cases, e.g., ζ -carotene has III>II, so that the value of %III/II is greater than 100. The weak absorbance around 320 nm shows information about cis structure.

HPLC has been the most widely used method for carotenoid analysis since the 1980's due to its high sensitivity, good separation, and short analysis times²⁵. HPLC chromatograms show retention time and peak area.

If HPLC is coupled with a photodiode-array (PDA) detector, it can record the liquid chromatogram at different wavelengths while scanning within a certain range of wavelengths to obtain UV spectra. This provides information about both HPLC retention times and UV characteristic absorbance. Under optimum conditions this method allows on-line identification of different peaks.

IR spectra show the characteristic absorbance of functional groups and are especially valuable for carbonyl containing compounds.

Mass spectrometry is an important technique that provides us both the molecular mass and a characteristic fragmentation pattern.

NMR is an important method for structure elucidation. It can provide proton coupling constants and chemical shift data to assign the location of protons on the carbon skeleton.

CD is one of the chiroptical methods and is used to determine the absolute configuration of stereocenters in chromophore, usually the method must be coupled with NMR data to fully establish the assignment²⁶.

K.Schiedt and S.Liaaen-Jensen²⁷ defined a minimum set of identification criteria for carotenoids. The criteria are: i) the UV/Vis spectrum (λ_{max} and fine structure) must agree with literature data; ii) chromatographic properties must be identical in TLC (R_f)

and HPLC (T_R), and the co-chromatogram with an authentic sample should be demonstrated; iii) a mass spectrum showing the molecular mass should be obtained. These are the minimum requirements for carotenoid identification.

Quantitative Analysis

UV/Vis is a commonly used method to analyze the concentration of pure carotenoids. The chromatographic techniques (HPLC) is the one most widely used methods to analyze the composition and concentration of carotenoids from natural extracts and fractions. The method of internal standards is particularly useful in HPLC because chromatographic results are not always reproducible from day to day.

Internal standards should be chemically similar to the analyte and should not react with any sample components. They should have similar chromatographic properties to the analyte and elute near the peaks of the analyte but should not coelute with them. Some internal standards used in carotenoid studies are: ethyloxime and methyloxime derivatives of β -apo-8'-carotenal^{28,29} and lutein alkyl ethers (3'-methyl lutein ether³⁰, 3'-ethyl lutein ether³¹ and 3'-hexyl lutein ether³²).

1.4 Outline of This Work

Studies have proven that lutein and zeaxanthin are two of the principal carotenoids in human serum³³ and the only carotenoids found in the retina³⁴. The zeaxanthin-lutein ratio dramatically changes with retinal eccentricity³⁰. The metabolism and transport of these carotenoids in the human body is still largely unknown. The understanding of the oxidation processes of hydroxy carotenoids is essential to the elucidation of the physiology of these molecules which appear to be essential for ocular health. In this work

zeaxanthin was oxidized *in vitro* with manganese dioxide, the oxidation products were isolated and purified using chromatographic methods including TLC, open column chromatography, and HPLC. The identification was performed by using TLC, HPLC retention time, UV/Vis, MS, IR, further confirmation was done by NMR.

3'-alkyl lutein ethers have been chosen as internal standards for the analysis of carotenoid concentrations in human serum, macular tissue, and monkey retina^{30,31,32} since they have similar stabilities and performance to carotenoids like lutein on the chromatographic systems. The extent to which these standards have been characterized is minimal consisting only of UV/Vis spectra and HPLC retention times. Their identity should be unambiguously established so that further work, dependent on these standards, is possible. In this work we prepared a series of 3'-alkyl lutein ethers by reacting lutein with corresponding acidified alkyl alcohols. The products were purified on HPLC, and characterized by HPLC retention time, UV/Vis, and mass spectra. The wide range of observed retention times provides a series of carotenoids suitable for use as internal standards.

Chapter II Oxidation of Zeaxanthin

2.1 Overview

Rhodoxanthin (4',5'-dehydro-4,5'-retro- β,β -carotene-3,3'-dione), as mentioned in Chapter I, is the main pigment of yew berries (*Taxus baccata*). For the structure of rhodoxanthin, Mayer et al. (1967)³⁵ assumed the natural rhodoxanthin to be 6, 6'-E (all-trans) (Fig. 2.1. A). This chemical structure profile was widely accepted by many researchers^{36, 37} until late 1970s, Andrewes et al.(1979)³⁸ and Englert et al.(1982)³⁹ verified the chemical structure of all-trans rhodoxanthin as B (Fig. 2.1.) via NMR. Englert et al. further separated all-trans rhodoxanthin and its two possible *cis*-isomers from synthetic all-trans rhodoxanthin by HPLC and deduced the structures of these two isomers as 6,6'-di-*cis* (Fig. 2.1. A, which were regarded as all-trans structures before) and 6-*cis* (Fig. 2.1. C).

Entschel et al.(1959)⁴⁰ first used the method of partial synthesis to produce rhodoxanthin from all-trans zeaxanthin *in vitro* with manganese dioxide. But only UV/Vis and IR were used to identify the products without LC, MS and NMR information. The complete characterization of the oxidation products of zeaxanthin remained a problem, the following is our work on this problem.

Fig. 2.1 Oxidation reaction of zeaxanthin and the structures of all-trans rhodoxanthin [B] and its 6,6'-di-*cis* [A], 6-*cis* [C] isomers.

2.2 Experimental

2.2.1 Reagents and Materials

Yellow maize (whole kernel yellow corn, previously frozen) was purchased from a local supermarket. All-trans zeaxanthin (synthetic) was a gift from Hoffmann-La Roche (Basel, Switzerland). Manganese (IV) oxide (MnO_2) (<5 micron, activated, ~85%) was purchased from Aldrich (Milwaukee, WI). Chloroform-d (99.8 atom % D) used in NMR

was purchased from ACROS (Geel, Belgium). The salt plate (IR crystal window, NaCl, undrilled, 25 mm D, 2 mm thick) was from Aldrich (Milwaukee, WI). All the other chemicals were analytical grade and solvents were HPLC grade or Optima purchased from Fischer Scientific (Fair Lawn, NJ) unless otherwise noted.

2.2.2 Instrumentation

High-performance Liquid Chromatography (HPLC)

The reversed-phase HPLC (R-HPLC) system consisted of a Rheodyne 7125 injector valve with 50 or 500 μ l loop, a LDC Analytical *constaMetric* 3200 solvent delivery system and a LDC/Milton Roy *spectroMonitor* D variable wavelength detector. A 50 μ l loop was used for the analytical column and a 500 μ l loop was for the preparative column. Three R-HPLC systems (I, II, III) were used.

System I: an analytical column Phenomenex Ultracarb 3 μ m ODS column (250 $\times$ 4.6 mm) (Torrance, CA) (Column I) coupled with a Phenomenex SecurityGuard C18 cartridge (4 $\times$ 3.0 mm) was used. The eluent was an isocratic mixture of acetonitrile-methanol (85:15, v/v) containing 1 ml triethylamine per liter of solvent to prevent degradation and improve separation of the carotenoids. The flow rate was 1 ml/min and the injection volume was 20 μ l.

System II: a newer analytical column (Column II), the same type and size of column as in System I coupled with the same guard column was used. A different ratio of eluent was used (acetonitrile:methanol:triethylamine, 90:10:0.1, v/v) to improve resolution. Other conditions were the same as in System I.

System III: a preparative column, Phenomenex Ultracarb 3 μm ODS column (250 $\times$ 10.0 mm) (Column III) coupled with the same guard column as above was used. The eluent was the same as in System II. The flow rate was set at 3.0 ml/min and the injection volume was 200 μl for the preparative column.

The normal-phase HPLC system consisted of a Rheodyne 8125 injector valve with 50 μl loop, a LDC/Milton Roy CM4000 multiple solvent delivery system and a SM4000 programmable wavelength detector. Two N-HPLC systems (IV and V) were used.

System IV: a Phenomenex IB-SIL 5 μm CN column (250 $\times$ 4.6 mm) coupled with a Phenomenex SecurityGuard CN cartridge (4 $\times$ 3.0 mm) was used. The eluent was a mixture of hexane, isopropyl acetate and acetone (76:8.5:3.5, v/v) with 1 ml of triethylamine per liter of solvent. The flow rate was 1.0 ml/min and the injection volume was 20 μl .

System V: a Phenomenex Prodigy 5 μm Silica 100 A (250 $\times$ 2.0 mm) without a guard column was used. The eluent was Hexane:Acetone:triethylamine (90:10:0.1, v/v), the flow rate was 0.8 ml/min and the injection volume was 20 μl .

The detection wavelength for both reversed-phase and normal-phase HPLC systems was set at 451 nm. All the HPLC data were recorded and processed using PeakSimple for Windows 95 (V 1.39), SRI Instruments, Torrance, CA.

UV/Visible Spectroscopy (UV/Vis)

The UV/Vis system was a Shimadzu UV-2101 PC UV-VIS double beam scanning spectrophotometer with UV-2101/3101 PC personal spectroscopy software (V2.0). Samples were dissolved in ethanol in order to obtain spectra which could be compared

with those in literature. 1 cm sample cuvettes were used. The spectrum was obtained scanning from 600-300 nm.

HPLC Coupled with Photodiode-Array Detector (PDA)

The HPLC-UV-PDA system (System VI) consisted of a Finnigan MAT SpectraSystem P4000 pump system, a AS3000 autosampler and a SpectraSystem UV6000LP photodiode-array detector. The newer analytical C₁₈ Column (column in system II) was used for this work. The eluent was acetonitrile:methanol:triethylamine (90:10:0.1, v/v) with 0.8 ml/min flow rate to guarantee the best possible resolution. The sample volume injected was 20 µl. The wavelength was monitored at 451 nm, the scan wavelength range was 600-300 nm with a 1 nm step, the scan rate was 5 Hz. Data were recorded and processed by ChromQuest (V2.1, ThermoQuest Finnigan Corporation, San Jose, CA).

Mass Spectrometry (MS)

The MS system consisted of a Finnigan MAT SpectraSystem P4000 pump system, a Finnigan AS3000 autosampler, and a Finnigan Navigator aQa mass spectrometer equipped with both ESI (electrospray ionization) and APCI (atmospheric pressure chemical ionization) configurations. The software was MassLab 2.2 supplied by Finnigan (San Jose, CA). Positive-ion APCI mode was selected. The samples were HPLC pre-purified and redissolved in methanol. A 20 µl of sample was injected into the system by the autosampler, the mobile phase was methanol. The temperature of the chamber was set at 250 °C, the corona voltage was 10 mV. Each sample was run 5 min. The spectra were recorded within 50-700 m/z or 400-700 m/z according to different samples. Background (blank) was subtracted in order to obtain a clean spectrum.

Nuclear Magnetic Resonance Spectrometry (NMR)

The NMR system was a Bruker Spectrospin 400 MHz UltraShield system with Xwin-nmr software (Fallanden, Switzerland). The samples were extremely pure and dried on a vacuum pump for at least overnight to make sure all the solvent was removed. About 0.6 ml of CDCl_3 was used to dissolve the sample. 0.5 mg of carotenoid sample was enough for ^1H NMR, but for ^{13}C NMR, several milligrams (*ca.* 4 mg) was usually needed. The number of scans was set at 128 for ^1H NMR, other parameters were copied from the previous file in the machine. For ^{13}C NMR, small amounts of a carotenoid sample require a large number of scans to obtain suitable intensity of the signal; however, the noise increased as the number of scans, so another set of parameters in the machine which has been optimized to decrease the noise while increasing the signal was used. Around 90,000 scans were set and the sample was run about 18 hours.

Infrared Spectrometry (IR)

The IR system was a Nicolet Magna-IR 560 spectrometer with OMNIC FT-IR Macros V4.0 software provided by Nicolet (Madison, WI). The sample was dissolved in dichloromethane and transferred carefully by a Pasteur pipette to the surface of NaCl plate to obtain an even coverage under nitrogen. NaCl plate without sample or polystyrene film [1.5 mil (38.1 μ)] was used as the blank.

2.2.3 Procedures

Isolation of All-trans Zeaxanthin from Available Natural Plant Resources

Methods similar to those mentioned by G.Britton and T.W.Goodwin⁴¹ or Schiedt and Liaaen-Jensen²⁷ were used to extract zeaxanthin standard from maize. The general

procedures included homogenization, extraction, saponification, chromatographic separation and purification.

Yellow maize (1 pound, about 454 g) was put in a blender and 500 ml of warm methanol (60 °C) was added. The mixture was blended for 30 sec to obtain a fine puree. This puree was filtered through a fast speed filter paper (P8, Fisher Scientific, Pittsburgh, PA) using vacuum filtration. Three additional 400 ml aliquots of methanol were used to extract the corn until a white solid remained. The yellow filtrates were combined. Sodium hydroxide (6%, w/v) was added into the filtrate, and the solution was stirred in the dark overnight at room temperature. Three portions of 500 ml of dichloromethane were used to extract the saponified carotenoids with the addition of saturated sodium bicarbonate solution to avoid formation of an emulsion. The dichloromethane phase was separated with a separatory funnel, combined and dried over anhydrous sodium sulfate overnight. The extract was then filtered and dried using a rotary evaporator with a cool water bath. If the extracted carotenoids still contained corn oil (a common occurrence) after the first saponification reaction, the procedure described above was repeated once more and the corn oil was removed.

Preliminary separation of the carotenoids was performed using a standard open column chromatographic method which was developed in our lab ⁴². The column used was a Kontes PTFE-plugged column (41 I.D.×600 L mm, with fritted disc.) packed with a mixture of calcium carbonate, magnesium oxide and calcium hydroxide (29.5:6:5, mass ratio, 80 mesh sifted), the length of this absorbent mixture was around 15 cm to obtain good separation. The eluent was a mixture of hexane, chloroform and acetone (3:2.4:0.5, v/v). The column was wrapped with aluminum foil to avoid being directly exposed to the

light during elution. Lutein eluted first followed by zeaxanthin. The zeaxanthin band was collected and dried on the rotary evaporator.

The identity of the zeaxanthin sample was confirmed by HPLC system I and UV/Vis.

Oxidation of Zeaxanthin

Methods similar to Entschel et al.⁴⁰ ca. 1959 were used to perform the zeaxanthin oxidation reaction. All of the procedures were done in the dark or dim light and all the containers were wrapped with aluminum foil to avoid degradation and isomerization of carotenoids.

At first, the oxidation reaction was optimized by adjusting several factors, including the concentration of the zeaxanthin solution, the height of the MnO₂ column, and the flow rate of the column. The optimal concentration was 10 mg of zeaxanthin in 100 ml acetone. If the solution was too concentrated, the excess zeaxanthin would be adsorbed on the column instead of being oxidized. The optimal height of the MnO₂ column was about 35 mm (in a Pasteur pipette, with 5 mm ID). If the packing of the column was too short or too long, zeaxanthin would be under- or over-oxidized. The optimal flow rate was 1 drop per 25 seconds (ca. 3 ml/hr). It is easy for zeaxanthin to be over-oxidized if it stays on the column longer than 35 seconds.

According to the optimal conditions, about 1 g activated MnO₂ was packed into a Pasteur pipette plugged with a small piece of glass wool. 1 or 2 ml of acetone was added into the pipette to moisten the packing, all the air bubbles were carefully tapped out and an evenly packed column (35 L×5 ID mm) was obtained. 10 mg/100ml acetone of yellow zeaxanthin solution was added to the column and a cherry-red solution was obtained. Usually MnO₂ would remain active for about 3 hours. The cherry-red solution was

filtered through a fritted funnel and dried using a rotary evaporator with a cool room temperature water bath. The dried sample obtained was kept in the freezer for future purification.

In order to obtain an accurate estimate of the yield of rhodoxanthin isomers represented on HPLC chromatogram by Peaks P1 and P2, the following procedure was performed. 100 μ l of zeaxanthin solution (10 μ g/100 μ l acetone) was treated with MnO_2 as previously described and the resulting cherry-red solution was carefully collected, dried under nitrogen, and redissolved in 100 μ l methanol. 20 μ l of the solution was injected in the HPLC. The resulting areas on the HPLC chromatogram for P1 and P2 were compared to that obtained when 20 μ l of zeaxanthin solution (10 μ g/100 μ l methanol) was injected in the HPLC. The percent yields were calculated using the ratio of peak areas of P1 (or P2) to zeaxanthin after correction for extinction coefficient (the calculation formula is shown in the following result section).

Isolation of Rhodoxanthin

To isolate rhodoxanthin from the oxidation reaction mixture, both thin-layer chromatography (TLC) and open column chromatography were tried because these two techniques can deal with large amounts of sample. Preparative HPLC was used, too. The efficiency of isolation using these three methods was evaluated.

TLC

Before performing the isolation, a small amount of the oxidation reaction mixture and authentic all-trans zeaxanthin sample (provided by Hoffmann-La Roche, Ltd.) were developed on a small plate [Uniplate silica gel GF plate (Analtech, Inc. Newark, DE), 10 $\times$ 2.5 cm, 250 microns. The solvent used was a mixture of methylene chloride and

methanol (98:2, v/v)]. Rhodoxanthin was located according to the R_f value ⁴³. A sample of the oxidation reaction mixture was dissolved in acetone and transferred onto the same type of silica gel plate (10×20 cm) using a capillary. The same solvent mixture was used to elute the carotenoids. After development, the plate was taken out of the chromatographic tank and kept in the dark to let the solvent evaporate. The cherry-red rhodoxanthin band was scraped from the plate as soon as possible and dissolved in methylene chloride, the mixture was filtered through a funnel with a fritted disc and the collected solvent was dried on the rotary evaporator. The R-HPLC system I was used to evaluate the efficiency of the isolation carried out on TLC.

Open Column Chromatography

The isolation of rhodoxanthin from the oxidation reaction mixture was done using the same chromatographic method (open column chromatography) as was used to isolate zeaxanthin from natural sources. The yellow band of zeaxanthin eluted first followed by the cherry-red band of rhodoxanthin. Rhodoxanthin band was collected in four vials in sequence to make sure as pure a sample as possible would be obtained. The collected samples were dried and identified by R-HPLC System I.

Preparative HPLC

R-HPLC System III (with a preparative column) was used to isolate rhodoxanthin from the oxidation reaction mixture. Two peaks (P1 and P2) which eluted at a time consistent with rhodoxanthin were collected and identified by R-HPLC System I.

HPLC with Better Resolution

A different HPLC profile with better resolution was obtained if the sample of the oxidation reaction mixture was run on System II (a new analytical C₁₈ column with a

more polar solvent). Three peaks (p2, p3, p4) which have the locations near those of P1 and P2 on preparative HPLC chromatogram were each collected separately. A little peak (p1) which eluted just before p2 was collected as well. In addition, a peak (p0) whose concentration prominently increased in the old samples of the oxidation reaction mixture was collected. These peaks were dried on the rotary evaporator or under nitrogen and kept in the freezer for further identification.

Identification of Rhodoxanthin in the Oxidation Reaction Mixture

• Peaks (P1 and P2) purified from the Preparative HPLC system II

P1 and P2 were identified using R-HPLC system I, UV/Vis and MS. P2 was run on NMR (both ^1H NMR and ^{13}C NMR) and IR. All the methods used are described in the section on instrumentation. Zeaxanthin was run using the same methods in order to obtain basic information for comparison.

• Peaks (p0, p1, p2, p3, p4) purified from HPLC system III

Peaks (p0, p1, p2, p3, p4) were identified using R-HPLC system III, UV/Vis and MS.

p0, p2, p3, p4 were further identified on N-HPLC System IV and System V.

For system IV (CN column), chromatographic conditions similar to Englert et al.(1982)³⁹ were used. A home-made column (500×3.2 mm I.D.) packed with Spherisorb S 5-CN (mean particle diameter 5 μm) (Phase Separations) and a eluent consisted of n-hexane-isopropyl acetate-acetone (76:17:7) with a flow rate of 1.0 ml/min were used to separate all-trans rhodoxanthin and its two cis-isomers. All-trans rhodoxanthin, 6,6'-di-cis and 6-cis isomers eluted at 11.5, 14.0, 16.5 min, respectively. In our case, the column was a Phenomenex IB-SIL 5 μm CN column (250×4.6 mm), much shorter than theirs. When using the same solvent ratio, the retention times for p2, p3 and p4 were around 8

min. A less polar eluent [hexane:isopropyl acetate:acetone (76:8.5:3.5, v/v)] was used to lengthen the retention time.

The zeaxanthin oxidation reaction mixture was run on an HPLC-UV-PDA system (System VI) to obtain on-line UV/Vis information for the peaks which eluted from HPLC. The UV/Vis spectra obtained were compared to those using conventional UV/Vis.

Zeaxanthin was run using the same methods for comparison. All the methods used were described in the instrumentation section above.

2.3 Results and Discussions

All-trans Zeaxanthin Isolated from Maize

The R-HPLC chromatogram and UV/Vis spectrum of all-trans zeaxanthin isolated from maize are shown in Fig.2.2 and Fig.2.3.

Fig. 2.2. R-HPLC chromatogram of the extracted all-trans zeaxanthin ($T_R=11.375\text{min}$) [on R-HPLC System I (p.12)]

Fig.2.2 shows that the retention time (T_R) for the prominent peak was 11.375 min, which is identical to that of authentic zeaxanthin sample. The percentage peak area of zeaxanthin was 80%. Further purification by R-HPLC provided analytically pure samples.

Fig.2.3. UV-Vis spectrum of the extracted all trans-zeaxanthin from maize.
($\lambda_{\max}$ =426, 451, 478.8 nm, %III/II=23, in ethanol.)

Fig.2.3 shows that the absorption maxima ($\lambda_{\max}$) of the extracted zeaxanthin from maize were observed at 426, 451 and 478.8 nm, within experimental error of the literature data 425, 450, 478 nm in ethanol ²⁴. A weak peak at 336 nm indicated there may be a small quantity (~7% from HPLC chromatogram) of cis-isomers present in the natural sample as expected. The value of %III/II was 23, similar to that of β -carotene (%III/II=25) ²⁴.

The concentration of the extracted zeaxanthin was calculated according to the Beer-Lambert law. The molar extinction coefficient of zeaxanthin used was 140,900 l/mol.cm²⁴. About 1 mg of pure zeaxanthin was obtained from 1 pound (454 g) maize after the above isolation procedures.

Oxidation of Zeaxanthin

Williams et al. (1966)⁴⁴ proposed a possible mechanism of retro rearrangement of double bonds for the biosynthesis of eschscholtzxanthin (4',5'-didehydro-4,5'-retro- β,β -carotene-3,3'-diol). Britton (1976)⁴⁵ suggested a similar oxidation process in the biosynthesis of rhodoxanthin. So the possible mechanism which might be involved in the oxidation reaction of zeaxanthin was proposed as follows (Fig.2.4).

Fig.2.4. Possible mechanism for the partial synthesis of rhodoxanthin.

Fig.2.5 was the HPLC chromatogram of the zeaxanthin oxidation reaction mixture prior to purification. The chromatographic information obtained is summarized in Table 2.1.

Fig.2.5. HPLC (system I) chromatogram of freshly made oxidation product mixture prior to purification.

The peak areas of rhodoxanthin (P1 and P2) and zeaxanthin on the chromatograms were used to calculate the % yield of the oxidation reaction. The values of the molar extinction coefficient of zeaxanthin (ϵ_{zea}) and rhodoxanthin (ϵ_{rho}) are 140,900 l/mol.cm (at 450 nm) and 140,500 l/mol.cm (at 490 nm), respectively²⁴. According to the UV/Vis spectra of P1, P2, and zeaxanthin (Fig.2.17), the value of ϵ_{P1} and ϵ_{P2} at 450 nm were corrected to $\epsilon_{\text{P1}(450\text{nm})}=140,500/1.106=127,034$ l/mol.cm and $\epsilon_{\text{P2}(450\text{nm})}=140,500/1.333=105,401$ l/mol.cm. The % yields of P1 and P2 were calculated using the following formula.

$$\% \text{ yield of P1 (or P2)} = \frac{\text{Peak Area of P1 (or P2)} \times \epsilon_{\text{zea}} (450\text{nm})}{\text{Peak Area of Zeaxanthin Before Reaction} \times \epsilon_{\text{P1 or P2}} (450\text{nm})}$$

$$\% \text{ of Unreacted Zeaxanthin} = \frac{\text{Peak Area of Unreacted Zeaxanthin}}{\text{Peak Area of Zeaxanthin Before Reaction}}$$

Table 2.1. Chromatographic information from Fig.2.5.

Peak Name	P1	P2	Pzea
Color	orange	Cherry-red	Yellow
T _R (min)	9.308	9.791	10.866
Yield %*	11	37	30 (%unreacted zeaxanthin)

Note*: At optimal conditions.

Fig.2.5 and Table 2.1 show that peaks labeled P1 and P2 eluted at a time consistent with rhodoxanthin as literature reported ⁴³. The last and most prominent peak was confirmed as zeaxanthin after co-injecting with authentic zeaxanthin sample [T_R(zea)=10.866min]. Based on the color of the two peaks, P2, which was cherry-red, was initially assigned as all-trans rhodoxanthin, and P1, which was orange, was tentatively assigned as a corresponding cis-isomer. The percent yield and the percentage of unreacted zeaxanthin were calculated using the peak areas before and after the oxidation reaction. At optimal conditions, the percent yields of P1 and P2 were found to be 11% and 37%, respectively, with 30% of zeaxanthin unreacted.

Isolation of Rhodoxanthin

• TLC

The bands on a silica plate showed that there was a good separation between rhodoxanthin (red band) and zeaxanthin (yellow band), the R_f values for the red band and

yellow band were 0.79 and 0.47, respectively, being consistent with the results in the previous work in our laboratory⁴³.

Fig.2.6. HPLC chromatogram (system I) of the red band isolated by TLC.

The HPLC chromatogram of the red band isolated by TLC (Fig. 2.6) shows that TLC was able to separate rhodoxanthin from unreacted zeaxanthin, but unable to separate rhodoxanthin from its cis-isomer and other impurities. It is concluded that TLC was not suitable to use for the purification of rhodoxanthin from other oxidation products.

Open Column Chromatography

On the open column, the zeaxanthin (yellow/orange) band eluted before the rhodoxanthin (cherry-red) band. Fig.2.7 shows a typical HPLC chromatogram of the rhodoxanthin sample collected from the red band on the chromatographic column at different times. It shows that the sample contained more or less zeaxanthin and other impurities (depending upon fraction sampled). P1, P2 were not separated from each other

to a satisfactory extent. It is concluded that this technique does not provide the desired resolution, many impurities tend to co-elute with one another.

Figure 2.7. HPLC chromatogram (system I) of the rhodoxanthin sample after purification by the open chromatographic column.

Fig.2.8. HPLC Chromatogram of freshly made oxidation product mixture on preparative HPLC system III ($T_R(P1)=17.766\text{min}$, $T_R(P2)=18.433\text{min}$).

Fig.2.8 shows the HPLC chromatogram obtained using the preparative HPLC column (system III), it shows that the HPLC profile was similar to that on the analytical column (system I) (Fig.2.5), but the retention times of P1 and P2 [$T_R(P1)=17.766\text{min}$, $T_R(P2)=18.433\text{min}$] were longer than those on the analytical column.

P1 and P2 purified on the preparative HPLC system III were run on R-HPLC system I to check the purity (Fig.2.9).

Fig.2.9. HPLC Chromatograms of purified P1(left) and P2(right) on HPLC system I [$T_R(P1)=9.350\text{ min}$, $T_R(P2)=9.766\text{ min}$].

Figure 2.9 shows the HPLC chromatograms of P1 and P2 collected from the preparative column (system III), P1 and P2 were very pure. It is concluded that preparative HPLC is a suitable method used to efficiently isolate rhodoxanthin and its isomer from zeaxanthin oxidation reaction mixture. Although preparative HPLC can not deal with as large a sample as TLC and liquid column chromatography did, it can purify about 200 μl of sample for each injection with guaranteed resolution. When a 5 mg of

zeaxanthin was used as starting material, after the oxidation reaction and the performance of purification on preparative HPLC, the amount of rhodoxanthin (P2) collected was 0.1 mg, the percent yield is around 2%; the percent yield of P1 is even lower than 1%. This technique was used in this work to obtain a rhodoxanthin sample (*ca.* 4 mg) pure enough to run ^1H -NMR and ^{13}C -NMR.

HPLC with Better Resolution

A different HPLC profile (Fig.2.10) is obtained when using HPLC system II with a newer C18 analytical column and a more polar eluent. The retention times were increased as expected. The peak (p2) which was labeled as P1 from the previous chromatogram (Fig.2.5) was not changed, however, P2 in Fig 2.5 was split into two peaks (now labeled as p3 and p4).

Fig.2.10. HPLC chromatogram of a freshly prepared sample of the zeaxanthin oxidation reaction mixture on system II. [$T_R(\text{p0})=3.841\text{min}$, $T_R(\text{p1})=9.416\text{min}$, $T_R(\text{p2})=10.125\text{min}$, $T_R(\text{p3})=10.725\text{min}$, $T_R(\text{p4})=11.051\text{min}$, $T_R(\text{zea})=13.358\text{min}$.]

Interestingly, if a sample of the zeaxanthin oxidation reaction mixture was kept in solvent in the freezer for a couple of weeks, the proportion of a peak (labeled as p0) which eluted at 3.808min significantly increased (Fig.2.11).

Fig.2.11. HPLC chromatogram of the sample (four weeks old) of zeaxanthin oxidation reaction mixture on system II.

In addition, if the samples of purified p1, p2, p3, and p4 were kept in a freezer for about four weeks and rerun on HPLC (system II), they isomerized into one another and decomposed to produce other compounds detectable on the HPLC. Among these, p0 is the most prominent. (Fig.2.12-2.15).

Fig.2.12. HPLC chromatogram of four week old sample of purified p1 on system II.
 $[T_R(p0)=3.758\text{min}, T_R(p1)=9.416\text{min}; T_R(p2)=10.033\text{min}, T_R(p3)=10.500\text{min},$
 $T_R(p4)=10.941\text{min}; \% \text{ peak area } (p1)=63\%]$

Fig.2.13. HPLC chromatogram of four week old sample of purified p2 on system II.
 $[T_R(p0)=3.766\text{min}, T_R(p1)=9.466\text{min}, T_R(p2)=10.133\text{min}, T_R(p3)=10.608\text{min}, T_R(p4)=$
 $10.990\text{min}; \% \text{ peak area } (p2)=64\%.]$

Fig.2.14. HPLC chromatogram of four week old sample of purified p3 on system II.
 $[T_R(p0)=3.766\text{min}, T_R(p2)=9.975\text{min}, T_R(p3)=10.616\text{min}, T_R(p4)=10.980\text{min};$
 $\% \text{ peak area (p3)}=58\%.]$

Fig.2.15. HPLC chromatogram of four week old sample of purified p4 on system II.
 $[T_R(p0)=3.783\text{min}, T_R(p1)=9.441\text{min}, T_R(p2)=10.105\text{min}, T_R(p3)=10.641\text{min},$
 $T_R(p4)=10.988 \text{ \& } 11.075 \text{ min}; \% \text{ peak area (p4)}=78\%.]$

Fig.2.12-2.15 show that purified p1, p2, p3, and p4 were unstable if kept in freezer for several weeks, they undergo isomerization and decomposition. It seems that p0 may be one of the main decomposition products of rhodoxanthin. p0 was collected as well as p1 (the little peak eluted before p2), p2, p3, and p4, rerun on the same HPLC system (system II) to check purity (Fig.2.16). They were found to be very pure after isolation on HPLC system II.

Fig.2.16. HPLC chromatograms of purified p0, p1, p2, p3, and p4 on system II.
[$T_R(p0)=3.83$ min, $T_R(p1)=9.34$ min, $T_R(p2)=10.22$ min, $T_R(p3)=10.82$ min,
 $T_R(p4)=11.15$ min.]

Analytical HPLC system II is the only method used to separate these peaks with high purity, but the efficiency is not satisfactory, the amount of each peak collected per day was around 0.01 mg. So in this work we were not able to obtain a large enough quality of very pure p1, p2, p3 and p4 to do NMR under the conditions available in our laboratory.

Identification of Rhodoxanthin

• P1 and P2

P1 and P2 isolated from the preparative HPLC column were identified using R-HPLC system I, UV/Vis, and MS. P2 was further studied by IR and NMR, but IR and NMR was not possible for P1 because of the lack of sufficient pure sample. R-HPLC chromatograms of purified P1 and P2 were shown in Fig.2.9. Other spectra were as follows. The spectrum of zeaxanthin is shown for comparison.

Fig.2.17. UV/Vis spectra of purified P1, P2, and synthetic zeaxanthin in ethanol.
[$\lambda_{\text{max}}(\text{P1})=491.4 \text{ nm}$, $\lambda_{\text{max}}(\text{P2})=497.0 \text{ nm}$, $\lambda_{\text{max}}(\text{zea})=452.0, 480.0 \text{ nm}$.
%III/II(P1, P2)=0, %III/II(Zea)=28]

UV/Vis spectra of purified P1 and P2 show that the wavelength of maximum absorption (λ_{max}) is greater than that of zeaxanthin, as expected because rhodoxanthin has 14 conjugated double bonds (c.d.b.s) while zeaxanthin only has 10 cdb.s. Zeaxanthin

gave a %III/II value of 28, similar to that in literature²⁴ (%III/II=25). P1 and P2, which show a broad peak without fine structure, are consistent with that of authentic rhodoxanthin isolated from yew berries as reported in the literature⁴³. The spectra of both P1 and P2 show a weak peak around 330 nm which is consistent with a *cis* structure. Additional work is required to unambiguously identify and characterize P1 and P2, these data are consistent with the assignment of these compounds as keto carotenoids.

Mass spectra are shown in Fig.2.18. APCI (atmospheric pressure chemical ionization) is a soft technique in which the chemical ionization of the mobile phase is achieved by utilizing corona discharge at atmospheric pressure. R.B.van Breemen, et al (1996)^{46, 47} first applied APCI LC/MS in carotenoid analyses. But only a few researchers^{48, 49} have used this method, and the application has been limited to the elucidation of known carotenoids like lutein, β -carotene, and lycopene. R.B.van Breemen, et al⁴⁶ observed the protonated molecule $[M+H]^+$ and molecular ion M^+ for both xanthophyll (lutein) and hydrocarbon carotenes (β -carotene) in positive-ion APCI. Furthermore, lutein showed a base peak at m/z 551 which corresponded to loss of a water from protonated molecule $[MH-18(H_2O)]^+$, in some cases, $[MH-18-92]^+$ was observed at m/z 459 corresponding to loss of a water and a toluene from protonated lutein. β -carotene showed only a weak peak at m/z 445 corresponding to loss of a toluene $[MH-92]^+$. T.Hagiwara (1998)⁴⁸ reported APCI spectrum of β -carotene only gave the protonated molecule at m/z 537. T.Lacker et al.(1999)⁴⁹ mentioned that the positive ion APCI spectra of both β -carotene and lycopene showed $[M+H]^+$, M^+ , and $[MH-92]^+$.

Similar methods were used to obtain mass spectra in this work.

Fig.2.18. Positive ion APCI mass spectra of zeaxanthin (upper, $[M+H]^+=569$), P1 (medium, $[M+H]^+=563$), and P2 (lower, $[M+H]^+=563$).

Mass spectrum of zeaxanthin (Fig.2.18) shows a base peak at m/z 569 which belongs to the protonated zeaxanthin ion $[M+H]^+$. Another ion recorded at m/z 551 is a typical fragment ion for loss of a water from protonated zeaxanthin $[MH-18]^+$. The three structures of rhodoxanthin (Fig.2.1) do not have hydroxyl groups which would favor the loss of a water, they should be more thermally stable than zeaxanthin and give less fragmentation. The spectra of P1 and P2 both give base peaks at m/z 563 corresponding to the protonated rhodoxanthin molecule. The spectrum of P2 shows a rather abundant peak at m/z 515 with a 48 mass difference from the base peak. It is hard to explain it as a fragment of rhodoxanthin, in some other cases this peak is not that prominent, probably it is a trace impurity or an instrumental artifact. No molecular ions M^+ were observed in the spectra of zeaxanthin, P1 and P2.

The infrared spectrum of zeaxanthin showed a broad absorption around 3330 cm^{-1} owing to the intermolecular hydrogen bonds of the hydroxyl groups on C3 and C3' positions while that of P2 showed a strong absorption band around 1660 cm^{-1} which is consistent with the absorption of the C=O stretching vibrations of rhodoxanthin in the literature⁴⁰.

^1H -NMR and ^{13}C -NMR spectra of synthetic all-trans zeaxanthin show in Fig.2.19 and Fig.2.20.

Fig.2.19. ¹H-NMR of synthetic all-trans zeaxanthin.

Fig.2.20. ¹³C-NMR of synthetic all-trans zeaxanthin.

Table 2.2 Experimental and literature ¹H-NMR data of all-trans zeaxanthin.

protons	δ (ppm)/No. H	Experimental	Literature at 400MHz (G.Englert ⁵⁰)
H ₃ C(16, 16'), H ₃ C(17, 17')		1.09, 1.14	1.074 / 6
Hax-C(2, 2')		1.47, 1.50, 1.53	1.48 / 1
Heq-C(2, 2')		1.70, 1.75, 1.77, 1.79	1.77 / 1
H ₃ C(18, 18')			1.74 / 3
H ₃ C(19, 19')		1.80, 1.99	1.97 / 3
H ₃ C(20, 20')			1.97 / 3
Hax-C(4, 4')		2.03, 2.06, 2.07, 2.10	2.04 / 1
Heq-C(4, 4')		2.38, 2.39, 2.42, 2.43	2.39 / 1
Hax-C-OH(3, 3')		4.02 (multiplet)	4.01 (broad) / 1
H-C(7, 7')		6.13, 6.14, 6.18, 6.19	6.11 / 1
H-C(8, 8')			6.13 / 1
H-C(10, 10')			6.16 / 1
H-C(11, 11')		6.28, 6.36, 6.40, 6.63, 6.64, 6.65, 6.66, 6.67, 6.70	6.64 / 1
H-C(12, 12')			6.36 / 1
H-C(14, 14')			6.25 / 1
H-C(15, 15')			6.63 / 1
		7.28 (caused by solvent) ⁵¹	

Zeaxanthin has a symmetric structure, so the assignment of the peaks on NMR spectra is also symmetric. It is however very difficult to assign the location of each proton on ¹H-NMR spectrum. The peak at 7.28 ppm was caused by the residual proton in the solvent (CDCl₃) and this value is identical to the data in the literature (δ=7.26 ppm)⁵¹. A broad peak around 4.02 ppm was assigned to the axial proton at C(3, 3'). Peaks around 1 to 2 ppm were assigned to the protons on saturated carbons [C(2, 2'), C(4, 4'), C(16, 16'), C(17, 17'), C(18, 18'), C(19, 19'), C(20, 20')]. Those around 6 ppm were contributed by the olefinic protons on the double carbon bonds [C(7, 7'), C(8, 8'), C(10, 10'), C(11, 11'), C(12, 12'), C(14, 14'), C(15, 15')]. The data were consistent with those in the literature⁵⁰.

Table 2.3 Experimental and literature ^{13}C -NMR data of all-trans zeaxanthin.

carbon	δ (ppm)	Experimental	Literature at 400MHz (G.Englert ⁵⁰)
C(20, 20')		13.188	12.8
C(19, 19')		13.249	
C(18, 18')		22.061	21.6
C(16, 16')		29.138	28.8
C(17, 17')		30.675	30.3
C(2, 2')		37.454	37.2
C(5, 5')		42.932	42.7
C(3, 3')		48.786	48.7
C(4, 4')		65.498	65.2
C(11, 11')		125.339	125.0
C(7, 7')		125.986	125.7
C(6, 6')		126.589	126.3
C(15, 15')		130.488	130.2
C(10, 10')		131.728	131.4
C(14, 14')		133.024	132.6
C(9, 9')		136.113	135.7
C(13, 13')		136.908	136.5
C(12, 12')		137.975	137.7
C(1, 1')		138.150	137.9
C(8, 8')		138.914	138.6

20 peaks were obtained from the ^{13}C -NMR spectrum of all-trans zeaxanthin, their chemical shifts were perfectly matched those in the literature⁵⁰.

Fig.2.21. ¹H-NMR of P2.

Fig.2.22. ^{13}C -NMR of P2.

Table 2.4. Experimental ^1H -NMR data of P2 and literature data of rhodoxanthin.

Δ (ppm) Protons	Experimental	Literature at 400MHz (G.Englert ^{39,50})		
		all-trans	6- <i>cis</i>	6,6'-di- <i>cis</i>
	0.896, 0.912, 0.918, 0.937, 0.956 (impurities)			
H ₃ C(16, 17)		1.387	1.251	1.251
H ₃ C(16', 17')			1.386	
H ₃ C(20)	1.2—2.4 (multiplet)	1.995	1.987	1.985
H ₃ C(20')			1.993	
H ₃ C(19)		2.029	2.016	2.017
H ₃ C(19')			2.029	
H ₃ C(18)		2.155	2.304	2.305
H ₃ C(18')			2.154	
H-C(2)		2.395	2.340	2.341
H-C(2')			2.394	
	4.2 (impurities)			
H-C(4)	5.955	5.936	~5.937	5.940
H-C(4')			~5.937	
H-C(12)	6.280 (multiplet)	6.260	6.242	6.238
H-C(12')			6.255	
H-C(14, 14')	6.400 (multiplet)	~6.43*	~6.43*	~6.43*
H-C(15, 15')				
H-C(10)	6.463 (multiplet)	6.462	6.404	6.402
H-C(10')			6.456	
H-C(11)	6.7—7.0 (multiplet)	6.785	6.739	6.739
H-C(11')			6.781	
H-C(8)		6.802	6.551	6.551
H-C(8')			6.800	
H-C(7)		6.904	6.667	6.668
H-C(7')			6.903	
	7.285 (caused by solvent) ⁵¹			
	7.5 and 7.7 (multiplet) (caused by impurities)			

* Center of gravity of the corresponding AA'BB'-type spectrum.

Among the three structures of rhodoxanthin (Fig.2.1) in literature, all-trans and 6,6'-di-*cis* rhodoxanthin are symmetric, half as many peaks are observed in the ^1H -NMR spectrum as compared to that of the asymmetric 6-*cis* rhodoxanthin. The ^1H -NMR

spectrum of P2 is far more complicated than the other three rhodoxanthin structures reported in the literature, in the later section we find out P2 is a mixture of all-trans rhodoxanthin and its cis isomer, this helps explain why the spectrum is so complex. There were three peaks which might have been contributed by impurities: one was around 4.21 ppm which is characteristic peak of an alcohol; another two were around 7.53 and 7.70 ppm. The sample of P2 had been checked for purity on HPLC before running the NMR and it was very pure. These contaminant peaks have been observed in the spectra of other samples run on the same instrument (instrumental artifact). They may come from the NMR tube. In our case, we did run NMR for the second time by removing the P2 sample from the NMR tube, drying down and transferring to a new tube, but this time the impure peaks remained. We were not able to collect another 4 mg sample of P2 to repeat the NMR test. The peaks around 0.2-2.4 ppm belong to the protons on C(2, 2'), C(16, 16'), C(17, 17'), C(18, 18'), C(19, 19'), C(20, 20'); the peak observed at 5.9 ppm was contributed by the protons on C(4, 4'); those around 6.3-7.0 ppm were assigned to the olefinic protons on double carbon bonds [C(7, 7'), C(8, 8'), C(10, 10'), C(11, 11'), C(12, 12'), C(14, 14'), C(15, 15')]. The peak at 7.28 ppm was the solvent peak as mentioned before.

The ^{13}C -NMR spectrum shows P2 is not a symmetric compound like all-trans or 6,6'-di-cis which gives only 19 peaks in the spectrum, neither is the asymmetric 6'-cis because it gives one peak at 141.8 ppm for C(9, 9') and another peak at 143.0 ppm for C(6, 6') while 6'-cis gives two separate peaks for C9 and C9', C6 and C6'. But it is a rhodoxanthin derivative with two characteristic peaks (199.38, 199.64 ppm) at C(3, 3') (Please turn to p.47.)

Table 2.5. Experimental ^{13}C -NMR data of P2 and literature data of rhodoxanthin.

δ (ppm) carbon	Experimental	Literature at 400MHz (G.Englert ^{39,50})		
		all-trans	6-cis	6,6'-di-cis
C(19, 19')	11.382, 12.829, 13.330, 14.436, 14.500	12.41	12.42	12.42
C(20, 20')		12.91	12.90	12.88
C(18)	22.787, 23.119, 23.410, 24.124, 25.830	22.31	25.46	25.42
C(18')			22.40	
C(16, 17)	28.739, 29.326, 30.125, 30.253, 30.378, 30.745	29.87	28.30	28.30
C(16', 17')			29.80	
C(1)	39.099	38.56	41.26	41.26
C(1')	38.914		38.44	
C(2)	52.947	54.37	52.44	52.50
C(2')	54.638, 54.702		54.22	
	68.551 (impurity)			
C(4)	126.357, 127.356, 128.647, 128.723, 129.214	126.05	125.91	128.83
C(4')			128.86	
C(11)		126.93	126.40	126.40
C(11')			125.91	
C(7)		128.15	125.48	125.48
C(7')			128.75	
C(8)		128.32	128.24	128.79
C(8')			128.24	
C(15)	130.018, 130.248	129.85	129.82	129.60
C(15')			129.56	
C(12)	131.326, 132.821, 132.876, 132.980	132.56	132.43	132.47
C(12')			132.56	
C(13)	137.947, 138.093, 138.138, 138.376, 138.438	137.51	137.92	137.54
C(13')			137.68	
C(14)		137.68	137.51	137.05
C(14')			137.48	
C(10)		138.04	138.37	137.87
C(10')			137.92	
C(9)	141.812	141.32	139.58	139.58
C(9')			141.40	
C(6)	142.995	142.70	143.80	143.83
C(6')			142.44	
C(5)	155.875	154.74	155.48	155.46
C(5')	155.371		154.99	
	168.212(impurity)			
C(3)	199.379, 199.638	198.95	199.25	198.99
C(3')			198.97	

consistent with carbonyl functional groups. The spectrum appears to be the result of a mixed sample, i.e. all-trans rhodoxanthin mixed with cis-isomer(s) and co-eluted on HPLC. It was not possible to make further assignments from the ^{13}C -NMR spectrum information. Once again, the peaks for impurities were still there with one prominent peak at 68.551 ppm and another at 168.212 ppm. The peak at 68.551 ppm is close to the characteristic location for carbons connected to a hydroxyl group, the peak at 168.212 ppm is usually contributed by the β position carbon in an α,β -unsaturated carbonyl group or the carbon in a carboxylic group. These data are not sufficient to resolve all the remaining questions and determine the structure.

The HPLC retention time, the absorption maxima (λ_{max}) and the fine structure from UV/Vis spectra, molecular mass and fragment information from mass spectrum obtained above meet the basic “minimum identification criteria” for carotenoids given by K.Schiedt and S.Liaaen-Jensen²⁷. From the information it is concluded that P1 and P2 are rhodoxanthin. Although we have NMR of P2, the information from NMR spectra were not sufficient to determine whether trans or cis structures should be assigned to P1 and P2. So P1 and P2 are characterized only as rhodoxanthin isomers. Further identity should be performed to obtain detail structure elucidation.

• p0, p1, p2, p3, and p4

The R-HPLC chromatograms of purified p0, p1, p2, p3, and p4 are shown in Fig.2.16.

Fig.2.23-2.26 shows the N-HPLC chromatograms of p0, p2, p3, and p4 on normal-phase CN column and silica column.

Fig.2.23. N-HPLC chromatograms of purified p0 on CN column (left, $T_R=10.01, 10.21$ min, a peak at 8.21 min is caused by dirty loop) and silica column (right, $T_R=5.66, 6.15$ min). Peak (p0) eluted from R-HPLC as one but separated into two peaks on N-HPLC.

Fig.2.24. N-HPLC chromatograms of purified p2 on CN column (left, $T_R=12.33, 13.33$ min) and silica column (right). P2 is a mixture.

Fig.2.25. N-HPLC chromatograms of purified p3 on CN column (left, $T_R=12.65$ min) and silica column (right, $T_R=5.52$ min). p3 shows only one peak on both R- and N-HPLC, it is a pure compound, not a mixture.

Fig.2.26. N-HPLC chromatograms of purified p4 on CN column (left, $T_R=13.66$ min) and silica column (right, $T_R=6.66$ min). p4 is a pure compound, not a mixture.

Fig.2.27 shows the HPLC chromatogram of zeaxanthin oxidation reaction mixture on HPLC-UV-PDA system (System VI), UV spectra obtained during this experiments are shown in Fig.2.29. The same sample was run on HPLC system II (Fig.2.28) for comparison.

Fig.2.27. HPLC chromatogram of zeaxanthin oxidation reaction mixture on HPLC-UV-PDA system (System VI).

Fig.2.28. HPLC chromatogram of zeaxanthin oxidation reaction mixture on System II for comparison.

Fig.2.29. UV/Vis spectra of p0, p1, p2, p3, p4, and zeaxanthin on HPLC-UV-PDA system (System VI) in 90:10:0.1 (v/v) of acetonitrile:methanol:triethylamine.

[$\lambda_{\text{max}}(\text{p0})=431 \text{ nm}$, $\lambda_{\text{max}}(\text{p1})=451, 477 \text{ nm}$ (a different profile, %III/II=127), $\lambda_{\text{max}}(\text{p2})=487 \text{ nm}$, $\lambda_{\text{max}}(\text{p3})=490 \text{ nm}$, $\lambda_{\text{max}}(\text{p4})=495 \text{ nm}$, $\lambda_{\text{max}}(\text{zea})=453.0, 480.5 \text{ nm}$ (%III/II=17).]

Fig.2.30. UV/Vis spectra of p0, p1, p2, p3, p4, and zeaxanthin in ethanol.
 $[\lambda_{\max}(\text{p0})=432.2 \text{ nm}, \lambda_{\max}(\text{p1})=489.8 \text{ nm}, \lambda_{\max}(\text{p2})=492.6 \text{ nm}, \lambda_{\max}(\text{p3})=496.6 \text{ nm},$
 $\lambda_{\max}(\text{p4})=497.7 \text{ nm}, \lambda_{\max}(\text{zea})=451.6, 479.2 \text{ nm}.]$

The chromatograms obtained on two R-HPLC systems (system VI and II) were very similar. UV spectra were used to compare with those obtained from conventional UV/Vis spectrophotometer (Fig.2.30). One of the advantages of HPLC coupled to a photodiode-array (PDA) detector is that the PDA can record the liquid chromatograms simultaneously at different wavelengths enabling UV spectra to be obtained for component peaks. This is a convenient method which allows on-line identification of different components.

The mass spectra of the blank (methanol), p0, p1, p2, p3, and p4 were shown in Fig.2.31-2.36.

Fig.2.31. Mass spectrum of the solvent (methanol) in MS system.

Fig.2.32. Mass spectrum of p0 (scan from 50-700 m/z). $[M+H]^+ = 351$.

Fig.2.33. Mass spectrum of p1. $[M+H]^+ = 563$.

Fig.2.34. Mass spectrum of p2. $[M+H]^+=563$.

Fig.2.35. Mass spectrum of p3. $[M+H]^+=563$.

Fig.2.36. Mass spectrum of p4. $[M+H]^+=563$.

Purified p0

The HPLC chromatogram of p0 (Fig.2.16) shows the retention time was 3.83 min, it is the most polar one of the five peaks collected from R-HPLC system II. As we can see from the HPLC chromatograms (Fig.2.11-2.15), the proportion of p0 increased in the zeaxanthin oxidation reaction mixture during storage; furthermore, p0 is the most prominent component when the purified peaks (p1, p2, p3, and p4) underwent degradation. So p0 appears to be a decomposition product of rhodoxanthin.

The UV/Vis spectrum of p0 (Fig.2.30) shows a rounded peak without fine structure ($\%III/II=0$), consistent with other conjugated ketocarotenoids. The wavelength of maximum absorption (λ_{max}) was 432.2 nm, about 60 nm less than that of rhodoxanthin. Based on comparison with similar systems in the literature²⁴, p0 has only 5 or 7

conjugated double bonds (c.d.b.). The weak peak at 330 nm suggests it is a compound with cis structure.

In the mass spectrum (Fig.2.32), the ion recorded at m/z 391 is a fragment from the solvent (Fig.2.31), the base peak observed at m/z 351 was assigned as the protonated ion.

From the information above, p0 was tentatively assigned as:

8 or 12-cis-13'-apo-3-keto retro dehydro- β -carotene-13'-al ($C_{24}H_{30}O_2$, MW=350).

There are 9 c.d.b.s in the compound not 5 or 7 as we mentioned above, the cis structure may influence the extent of conjugation in the system. The possible structures are shown as follows:

Fig.2.37. The possible structures of p0.

Purified p1

P1 shows different profiles of UV/Vis spectra on the PDA (Fig.2.29) and UV/Vis spectrophotometer (Fig.2.30). A spectrum ($\%III/II > 100$) identical to that obtained on PDA was also observed for one sample of p1 whose spectrum was obtained on the UV/Vis spectrophotometer. Other samples of p1 gave the spectrum shown in Fig.2.30. A

base peak at m/z 563 $[M+H]^+$ on mass spectrum shows a rhodoxanthin-like peak consistent with the characterization as a ketocarotenoid. $\lambda_{max}=489.8$ nm, 3 nm less than p2, 7 nm less than p3, and 8 nm less than p4. p1 appears to be a less conjugated cis structure. It is concluded that p1 is a rhodoxanthin cis-isomer.

Purified p2

P2 has a similar UV/Vis profile on both PDA and spectrophotometer. The N-HPLC chromatograms show a number of components are present in this sample. They are all keto compounds exhibiting no fine structure. A weak peak at 330 nm shows that they are cis compounds or at least mixed with cis compounds. The mass spectra show only a $[M+H]^+$ at m/z 563 identical to protonated rhodoxanthin. It is concluded that p2 is a mixture of rhodoxanthin cis-isomers.

Purified p3

p3 has a similar UV/Vis profile on both PDA and the spectrophotometer as well. The N-HPLC chromatogram confirms that this is a single compound not a mixture. It has a typical keto compound UV/Vis spectrum with no vibration fine structure. The spectrum also shows a weak cis peak at 330 nm. The mass spectrum shows $[M+H]^+$ peak at m/z 563 identical to the protonated rhodoxanthin. So p3 is a rhodoxanthin cis-isomer.

Purified p4

The shape of the UV/Vis spectrum of p4 is similar to those observed for p2 and p3, but the cis peak at 330 nm was very small. The N-HPLC chromatogram shows the presence of only one compound. The mass spectrum had a $[M+H]^+$ peak at 563. A comparison of the subjective color of peaks p1 through p4, p4 is red, others are cherry-

red or orange, suggested that p4 must be the all-trans rhodoxanthin compounds. Additional information will be needed for detailed characterization.

2.4 Conclusions

Rhodoxanthin was prepared by partial synthesis from all-trans zeaxanthin by an *in vitro* oxidation with manganese dioxide at about 40% yield. TLC and open column chromatography were not suitable for the separation of rhodoxanthin from the oxidation reaction mixture because they can not isolate rhodoxanthin and its isomers. Preparative reversed-phase HPLC was used to separate rhodoxanthin products. Two peaks (P1 and P2) were collected for further identification. HPLC retention time, UV/Vis, and MS information show P1 and P2 are rhodoxanthin isomers. Although the ^1H - and ^{13}N -NMR of P2 were obtained, it was not possible to fully characterize the sample. When using a newer analytical C18 column with more polar eluent, P2 was separated into two peaks (p3 & p4) and a different profile of the HPLC chromatogram was obtained. p0, p1, p2 (P1 before), p3 and p4 (P2 before) were collected for further characterization. R-HPLC, N-HPLC, UV/Vis, and MS information show p0 is a decomposition product of rhodoxanthin characterized as **8 or 12-cis-13'-apo-3-keto retro dehydro- β -carotene-13'-al** with 9 c.d.b.s. p1 is a rhodoxanthin cis isomer; p2 is a mixture of several rhodoxanthin cis-isomers; p3 is a rhodoxanthin isomer with cis structure. p4 appears to be all-trans rhodoxanthin. It was not possible to obtain an NMR on this compound because of the lack of sufficient sample. Additional experiments must be performed in order to fully characterize these compounds.

Chapter III Characterization of 3'-Alkyl Lutein Ethers

3.1 Experimental

3.1.1 Preparation of 3'-Alkyl Lutein Ethers

According to Liaaen-Jensen and Hertzberg (1966)⁵², the hydroxyl group on 3' allylic position in lutein is easily methylated by acidified methanol. 3'-alkyl lutein ethers were prepared by using the similar method in this study (Fig.3.1).

100 μ l of concentrated hydrochloric acid was added into 10 ml of each of different twelve alcohols, i.e. methanol, ethanol, isopropanol, n-propanol, sec-butanol, n-butanol, isobutanol, t-butanol, n-pentanol, n-hexanol, phenethyl alcohol (Eastman Kodak, Rochester, New York), and benzyl alcohol (J.T.Baker Chemical CO. Phillipsburg, New Jersey) (Table 3.1). The acidified alcohol was kept in freezer before use. 0.1 mg of synthetic lutein [(3R, 3'R, 6'R')- β,ϵ -carotene-3, 3'-diol, a gift from Kemin Foods, Inc.] was dissolved by adding the acidified alcohol solution slowly until all solid was in solution (about 5 ml). The reaction mixture was kept in the refrigerator overnight to ensure the reaction reached completion. 10 ml of deionized water was added to quench the reaction and 10 ml of saturated sodium bicarbonate solution was added to neutralize the products. The crude product mixture was extracted with dichloromethane using the same procedure described in Chapter 2 (p.16). Dichloromethane which contained unreacted alcohol was evaporated using a rotary evaporator with a room temperature water bath. The unreacted n-pentanol, n-hexanol, phenethyl alcohol, and benzyl alcohol were difficult to remove because of their high boiling points (Table 3.1), a vacuum pump

Fig.3.1. The etherification reaction of lutein and acidified alcohol (ROH).

was used to fully remove all residual alcohol. The melting point for t-butanol is 25.5 °C, it crystallizes if the temperature is lower than 25 °C, therefore the mixture of lutein and acidified t-butanol was kept at room temperature rather than in the refrigerator for the reaction.

Table 3.1. Alcohols and their corresponding lutein ethers.

No	Name of alcohol	Molecular Formula (ROH)	m.p./ b.p. (°C)*	Dielectric Constant(ϵ')	Expected product
1	Methanol	CH ₃ OH	-98 / 65	32.6	3'-methyl lutein ether
2	Ethanol	CH ₃ CH ₂ OH	/ 78	24.3	3'-ethyl lutein ether
3	Isopropanol	(CH ₃) ₂ CHOH	-89.5 / 82	18.3	3'-isopropyl lutein ether
4	n-propanol	CH ₃ CH ₂ CH ₂ OH	-127 / 97	20.1	3'-n-propyl lutein ether
5	t-butanol	(CH ₃) ₃ COH	25.5 / 82.2	10.9 (30)	3'-n-butyl lutein ether
6	sec-butanol	CH ₃ CH(OH)CH ₂ CH ₃	-115 / 98	15.8	3'-sec-butyl lutein ether
7	n-butanol	CH ₃ CH ₂ CH ₂ CH ₂ OH	-89.5 / 117	17.1	3'-n-butyl lutein ether
8	isobutanol	(CH ₃) ₂ CHCH ₂ OH	-108 / 107	17.7	3'-isobutyl lutein ether
9	n-pentanol	CH ₃ (CH ₂) ₄ OH	-79 / 137	13.9	3'-n-pentyl lutein ether
10	n-hexanol	CH ₃ (CH ₂) ₅ OH	-46.7 / 158	N/A	3'-n-hexyl lutein ether
11	phenethyl alcohol	C ₆ H ₅ CH ₂ CH ₂ OH	-27 / 218	4.22 (20)	3'-phenethyl lutein ether
12	benzyl alcohol	C ₆ H ₅ CH ₂ OH	-15 / 205	13.1 (20)	3'-benzyl lutein ether

Note: *m.p.—melting point; b.p.—boiling point. Data were from the label on alcohol container or from Aldrich catalog book.

Dielectric constant (ϵ') was at 25° C unless followed by another temperature in parentheses. Data were from “the Chemist’s Companion--A Hand Book of Practical Data, Techniques, and References” by A.J.Gordon and R.A.Ford. Wiley-Interscience, 1972.

The product of lutein and each alcohol was dissolved in methanol and purified by using R-HPLC. Here HPLC system I [with a C18 analytical column (Column1)] was used, the eluent and other conditions were exactly the same as described in Chapter 2 (p.12). The collected fractions were dried for further identification after checking the purity on the same R-HPLC system.

3.1.2 Characterization of 3'-Alkyl Lutein Ethers

Further identification of lutein ethers was done using UV/Vis spectrometry and mass spectrometry (MS). UV/Vis spectrum and Mass Spectrum were obtained using the same methods described in Chapter 2 (UV pp.13-14, MS p.14).

It was assumed that lutein and lutein ethers have the same molar extinction coefficients. The concentration of lutein ether was calculated according to the Beer-Lambert Law from the absorbance on UV/Vis spectrum and the molar extinction coefficient of lutein ($\epsilon = 144,800 \text{ l/mol.cm}$ in ethanol)²⁴. Identical molar amounts (*ca.* 1×10^{-9} moles) of selected lutein ethers were added together, this mixture was concentrated under nitrogen and dissolved in 500 μl of methanol. A combined chromatogram of the resulting mixture containing nine lutein ethers was obtained on the R-HPLC system I.

3.2 Results and Discussions

This reaction of lutein and acidified alcohol was regarded as a standard S_N1 reaction by C.H.Eugster (1995)⁵³. The hydroxyl group on the C3' allylic position of lutein was lost under the acidic conditions and a relatively stable allylic secondary carbocation formed. The alcohol, which was the nucleophile in this reaction, attacked the carbocation producing the 3'-alkyl lutein ether after loss of a proton. It was noted that the diastereoisomeric alkyl lutein ethers (*i.e.* 3'-alkyl lutein ether and 3'-epi-alkyl lutein ether) would be expected but this was not demonstrated. The mechanism⁵⁴ was as follows (Fig.3.2). Other possible products that might be obtained from the reaction were the *cis*-isomers on the polyene chain (*e.g.* 13-*cis*) and anhydrolutein (Fig.3.1).

As mentioned by C.H.Eugster (1995)⁵³, the methylated product of lutein (3'-methyl lutein ether) has an unchanged UV/Vis spectrum but is slightly less polar than lutein. These two stereoisomeric lutein ether products (3'-alkyl lutein ether and 3'-epi-alkyl lutein ether) were expected to have the same UV spectra as their parent compound--lutein

but their retention times were different from that of lutein. The following is what we have observed from HPLC, UV/Vis and MS of these alkyl ether derivatives.

Fig.3.2. Mechanism of the S_N1 reaction of lutein and acidified alcohol.

R-HPLC Chromatography

HPLC chromatograms of the reaction products obtained with the twelve different alcohols and lutein are shown in Table 3.2.

The chromatograms of two products (freshly made and five days old) of 3'-hexyl lutein ether were compared as follows.

Fig.3.3. HPLC chromatogram of freshly made 3'-hexyl lutein ether.
 Lut: lutein; Zea: zeaxanthin; 10: 3'-hexyl lutein ether 1; 10': 3'-hexyl lutein ether 2.
 $T_R(10)=44.266$ min, $T_R(10')=45.166$ min.

Fig.3.4. HPLC chromatogram of 5 days old sample of 3'-hexyl lutein ether.
 $T_R(10)=43.955$ min, $T_R(10')=44.983$ min.

Fig.3.3 shows the HPLC chromatogram of freshly made products obtained by reaction of lutein and acidified n-hexanol produced two peaks (10 and 10'). The retention times were 44.266 min and 45.166 min for peak 10 and peak 10', respectively. Peak #10' was seen to be a shoulder of peak #10. If the sample was kept in freezer and analyzed a few days later, the profile of the chromatogram did not change much [$T_R(10)=43.955$ min, $T_R(10')=44.983$ min] but the resolution of these two peaks improved (Fig.3.4). The following isomerization and dehydration may occur when trace amounts of H^+ are present in the reaction mixture during storage in the freezer.

Fig.3.5. Isomerization of lutein ether & epi-lutein ether and dehydration in the reaction mixture.

When the reaction mixture was kept in the freezer for about five days, an equilibrium between the two peaks of the reaction products appeared to result. Such old samples were used in purifying the two peaks from the other products to obtain the best efficiency.

Fig.3.6. HPLC chromatograms of purified 3'-hexyl lutein ether 1 (10) and 3'-hexyl lutein ether 2 (10'), they are very clean after purified by using R-HPLC.

Fig.3.7. HPLC chromatogram of 3'-methyl lutein ether.
 $T_R(1)=17.116$ min, $T_R(1')=17.766$ min.

Fig.3.8. HPLC chromatogram of 3'-ethyl lutein ether.
 $T_R(2)=20.233$ min, $T_R(2')=20.733$ min.

Fig.3.9. HPLC chromatogram of 3'-isopropyl lutein ether.
 $T_R(3)=22.75$ min, $T_R(3')=23.40$ min.

Fig.3.10. HPLC chromatogram of 3'-n-propyl lutein ether.
 $T_R(4)=24.966$ min, $T_R(3')=25.800$ min.

Fig.3.11. HPLC chromatogram of the reaction of lutein and t-butanol at room temperature. No lutein ether peak is available.

Fig.3.11 shows that there was no reaction occurred when lutein was mixed with acidified t-butanol. It is likely that t-butanol is easily protonated under the acidic condition and then undergoes loss of H₂O at rates that exceed those of the allylic hydroxyl group in lutein. The resulting cation would produce 2-methylpropene [CH₂=C(CH₃)₂]. Meanwhile, lutein could undergo dehydration under the acidic condition (Fig.3.5) and those peaks around 17 min to 23 min showed in Fig.3.11 may be the dehydrated lutein peaks. The t-butyl ether of lutein is therefore not readily prepared by this method.

Fig.3.12. HPLC chromatogram of 3'-sec-butyl lutein ether.
 $T_R(6)=27.566$ min, $T_R(6')=28.350$ min.

Fig.3.13. HPLC chromatogram of 3'-n-butyl lutein ether.
 $T_R(7)=29.233$ min, $T_R(7')=29.800$ min.

Fig.3.14. HPLC chromatogram of 3'-isobutyl lutein ether.
 $T_R(8)=29.783$ min, $T_R(8')=31.233$ min.

Fig.3.15. HPLC chromatogram of 3'-n-pentyl lutein ether.
 $T_R(9)=36.783$ min, $T_R(9')=37.700$ min.

Fig.3.16. HPLC chromatogram of expected 3'-phenethyl lutein ether.
 $T_R(11)=23.816$ min.

The profile of HPLC chromatogram of the expected 3'-phenethyl lutein ether shown in Fig.3.16 was different with those of other lutein ethers. Only one peak was obtained. The retention time was at 23.816 min, similar to that of the 3'-ethyl lutein ether ($T_R=22.233$ min), 3'-isopropyl lutein ether ($T_R=22.75$ min), and n-propyl lutein ether ($T_R=24.966$ min). The peak was collected for further identification.

Fig.3.17. HPLC chromatogram of expected 3'-benzyl lutein ether. No significant carotenoid peaks, either product or parent material lutein is observed.

No 3'-benzyl lutein ether was obtained after the treatment of lutein with acidified benzyl alcohol. Moreover, all the lutein was decomposed in this reaction under the conditions used.

Table 3.2. Information from HPLC chromatograms of lutein ethers.*

No.	Name of lutein ether	Molecular Formula (C ₄₀ H ₅₄ -3-OH-3'-OR)	T _R (min) for Lutein ether /Epi lutein ether
1	3'-methyl lutein ether	C ₄₀ H ₅₄ -3-OH-3'-OCH ₃	17.116 / 17.766
2	3'-ethyl lutein ether	C ₄₀ H ₅₄ -3-OH-3'-OCH ₂ CH ₃	20.233 / 20.733
3	3'-isopropyl lutein ether	C ₄₀ H ₅₄ -3-OH-3'-OCH(CH ₃) ₂	22.75 / 23.40
4	3'-n-propyl lutein ether	C ₄₀ H ₅₄ -3-OH-3'-OCH ₂ CH ₂ CH ₃	24.966 / 25.800
5	N/A		N/A
6	3'-sec-butyl lutein ether	C ₄₀ H ₅₄ -3-OH-3'-OCH(CH ₃)(CH ₂ CH ₃)	27.566 / 28.350
7	3'-n-butyl lutein ether	C ₄₀ H ₅₄ -3-OH-3'-OCH ₂ CH ₂ CH ₂ CH ₃	29.233 / 29.800
8	3'-isobutyl lutein ether	C ₄₀ H ₅₄ -3-OH-3'-OCH ₂ CH(CH ₃) ₂	29.783 / 31.233
9	3'-n-pentyl lutein ether	C ₄₀ H ₅₄ -3-OH-3'-O(CH ₂) ₄ CH ₃	36.783 / 37.700
10	3'-n-hexyl lutein ether	C ₄₀ H ₅₄ -3-OH-3'-O(CH ₂) ₅ CH ₃	44.266 / 45.166
11	3'-phenethyl lutein ether	C ₄₀ H ₅₄ -3-OH-3'-OCH ₂ CH ₂ C ₆ H ₅	23.816(only one peak)
12	N/A		N/A

Note: *Chromatographic conditions (System I): Column (Column I): a Phenomenex Ultracarb 3 μ m ODS column (250 $\times$ 4.6 mm) with Phenomenex SecurityGuard C18 cartridge (4 $\times$ 3.0 mm) guard column; eluent: acetonitrile-methanol-triethylamine (85:15:0.1, v/v); flow rate: 1 ml/min; detection wavelength: 451 nm; injection volume: 20 μ l.

We can see that the profiles for the HPLC chromatograms shown for the lutein ethers above are similar. The retention times (T_R) of the ethers are greater than that of lutein keeping with expectation (Table 3.2). The retention times for lutein ethers increase with the alkyl chain length and are inversely related to the polarity of the parent alcohols (Table 3.2). The percent yield of lutein ether from each reaction was calculated using the peak areas of lutein and lutein ether before and after the reaction. The % yields for most of the lutein ethers were higher than 80% of recovered carotenoid.

UV/Vis Spectrometry

The spectra of lutein, 3'-hexyl lutein ether 1, and 3'-hexyl lutein ether 2 in ethanol are shown in Fig.3.18.

Fig.3.18. UV/Vis spectra of lutein, 3'-hexyl lutein ether 1, and 3'-hexyl lutein ether 2 in ethanol.

Fig.3.18 shows the profiles of UV/Vis spectra of lutein, 3'-hexyl lutein ether 1, and 3'-hexyl lutein ether 2 were all identical. The absorption maxima (λ_{max}) for lutein were observed at 421.0, 445.2, and 474.2 nm, being consistent with the literature data of lutein (422, 445, 474 nm, in ethanol.); the related data for 3'-hexyl lutein ether 1 and 3'-hexyl lutein ether 2 were 421.0, 446.4, 475 nm and 420.8, 446.0, 475.0 nm, respectively. The values of %II/III for lutein, 3'-hexyl lutein ether 1, and 3'-hexyl lutein ether 2 were 51, 55, 52, respectively. There was a weak peak observed at 331 nm in the spectrum of 3'-hexyl lutein ether 2. This peak was not apparent in the spectra of lutein and 3'-hexyl lutein ether and is consistent with a cis-isomer component in the fraction.

Mass spectrometry (MS)

The mass spectra for lutein and 3'-alkyl lutein ethers were as follows.

Fig.3.19. Mass spectrum of lutein.

Fig.3.20. Mass spectrum of 3'-methyl lutein ether.

Fig.3.21. Mass spectrum of 3'-ethyl lutein ether.

Fig.3.22. Mass spectrum of 3'-isopropyl lutein ether.

Fig.3.23. Mass spectrum of 3'-n-propyl lutein ether.

Fig.3.24. Mass spectrum of 3'-sec-butyl lutein ether.

Fig.3.25. Mass spectrum of 3'-n-butyl lutein ether.

Fig.3.26. Mass spectrum of 3'-isobutyl lutein ether.

Fig.3.27. Mass spectrum of 3'-n-pentyl lutein ether.

Fig.3.28. Mass spectrum of 3'-n-hexyl lutein ether 1.

Fig.3.29. Mass spectrum of 3'-n-hexyl lutein ether 2.

Fig.3.30. Mass spectrum of the purified peak from the mixture of lutein and acidified phenethyl alcohol. There is no $[M+H]^+$ at m/z 673.

The information obtained from mass spectra was summarized in Table 3.3.

Table 3.3. Information from mass spectra of lutein ethers.

No	Name	Molecular weight	Characteristic peaks (m/z)
0	Lutein	568	569[M+H] ⁺ , 551[MH-H ₂ O] ⁺ , 459[MH-18-92] ⁺
1	3'-methyl LE	582	583[M+H] ⁺ , 551[MH-CH ₃ OH] ⁺ , 459[MH-32-92] ⁺
2	3'-ethyl LE	596	597[M+H] ⁺ , 551[MH-C ₂ H ₅ OH] ⁺ , 459[MH-46-92] ⁺
3	3'-isopropyl LE	610	611[M+H] ⁺ , 551[MH-C ₃ H ₇ OH] ⁺ ,
4	3'-n-propyl LE	610	611[M+H] ⁺ , 551[MH-C ₃ H ₇ OH] ⁺ ,
6	3'-sec-butyl LE	624	625[M+H] ⁺ , 551[MH-C ₄ H ₉ OH] ⁺ , 459[MH-74-92] ⁺
7	3'-n-butyl LE	624	625[M+H] ⁺ , 551[MH-C ₄ H ₉ OH] ⁺ , 459[MH-74-92] ⁺
8	3'-isobutyl LE	624	625[M+H] ⁺ , 551[MH-C ₄ H ₉ OH] ⁺ ,
9	3'-n-pentyl LE	638	639[M+H] ⁺ , 551[MH-C ₅ H ₁₁ OH] ⁺ ,
10	3'-n-hexyl LE	652	653[M+H] ⁺ , 551[MH-C ₆ H ₁₃ OH] ⁺ , 459[MH-102-92] ⁺
10'	3'-epi-n-hexyl LE	652	653[M+H] ⁺ , 551[MH-C ₆ H ₁₃ OH] ⁺ , 459[MH-102-92] ⁺
11	3'-phenethyl LE	672	No [M+H] ⁺ , has 551, 531(?)

The profiles of the mass spectra for lutein and nine lutein ethers (Fig.3.19-3.29, #1-#4 and #6-#10' in Table 3.3) were similar. Lutein shows [M+H]⁺ at m/z 569, [MH-18]⁺ at m/z 551; all of the nine lutein ethers show protonated molecules [M+H]⁺ at corresponding m/z and a fragment ion at m/z 551 which corresponds to loss of an alcohol from protonated molecule {[MH-ROH]⁺}. Molecular ions {M⁺} were not observed which is different as in the literature^{46, 47, 49}. The mass spectra of 3'-hexyl lutein ether 1 (Fig.3.28) and 3'-hexyl lutein ether 2 (Fig.3.29) were identical.

Fig.3.30 shows there was no [M+H]⁺ peak at m/z 673 corresponding to the expected 3'-phenethyl lutein ether, although there was a fragment ion at m/z 551 as was observed for lutein and the other lutein ethers. It is concluded that the sample is not 3'-phenethyl

lutein ether but is clearly a lutein derivative. Further study will be required to identify this reaction product.

HPLC chromatogram of nine 3'-alkyl lutein ethers

Fig.3.31. Combined HPLC chromatogram of nine 3'-alkyl lutein ethers.

The chromatographic conditions were the same as listed in Table 3.2.

1: 3'-methyl lutein ether; 2: 3'-ethyl lutein ether; 3: 3'-isopropyl lutein ether; 4: 3'-n-propyl lutein ether; 6: 3'-sec-butyl lutein ether; 7: 3'-n-butyl lutein ether; 8: 3'-isobutyl lutein ether; 9: 3'-n-pentyl lutein ether; 10: 3'-n-hexyl lutein ether. Please refer to Table 3.4 for detail.

Fig.3.31 and Table 3.4. show that the retention times for nine 3'-alkyl lutein ethers were, as expected, close to those obtained from the corresponding chromatogram of individual lutein ethers. The resolution for most of the lutein ethers is excellent excepting 3'-n-butyl lutein ether (#7) and 3'-isobutyl lutein ether (#8). The retention times for lutein ethers increase as with the chain length and are consistent with a more lipophilic nature of the ethers with increasing length of the alkyl groups.

Table 3.4. Information from the combined HPLC chromatogram of lutein ethers.

No.	Name of lutein ether	T _R (min) for Lutein ether	Peak area
1	3'-methyl lutein ether	17.566	5670
2	3'-ethyl lutein ether	20.616	4830
3	3'-isopropyl lutein ether	22.916	5336
4	3'-n-propyl lutein ether	25.083	5411
6	3'-sec-butyl lutein ether	28.083	5335
7	3'-n-butyl lutein ether	30.183	10797 (combined area for peak 7 and peak 8.)
8	3'-isobutyl lutein ether	30.566	
9	3'-n-pentyl lutein ether	36.450	5361
10	3'-n-hexyl lutein ether	44.766	4316

3.3 Conclusions

Nine 3'-alkyl lutein ethers (alkyl = methyl, ethyl, isopropyl, n-propyl, sec-butyl, n-butyl, isobutyl, n-pentyl, n-hexyl) were prepared in good yields by reacting lutein with corresponding acidified alkyl alcohols.

HPLC chromatograms show a ~50:50 mixture of two 3'-alkyl lutein ether isomers are obtained. The two resulting isomers (two components) were isolated by reversed-phase HPLC with satisfactory purity. These two components were initially thought to be the 3'R and 3'S stereoisomers of the allylic alkyl ethers of lutein. The first one in the chromatogram was thought to be 3'R-alkyl lutein ether, and the second, 3'S-epi-alkyl lutein based on the observation that lutein and epilutein diastereomers separate in the same relative order. The retention times for lutein ethers increase with the alkyl chain length.

All of the 3'-alkyl lutein ethers showed the same UV/Vis spectrum as that of lutein. There is an obvious weak peak at 331 nm in the spectrum of the second component which is consistent with a *cis* structure, this is in conflict with the previous assignment as the epilutein ether.

Alkyl lutein ethers produced protonated molecular ions $[M+H]^+$ and fragment ions $[MH-ROH]^+$ in the positive ion APCI mass spectra, in a pattern similar to lutein. There is no difference in the mass spectra of the two 3'-alkyl lutein ether isomers.

HPLC retention time, UV/Vis, and MS reported here meet the basic "minimum identification criteria" for carotenoids given by K. Schiedt and S. Liaaen-Jensen²⁷. Based on these data, the first peak was identified as a mixture of 3'R and 3'S-alkyl lutein ether diastereoisomers, and the second peak was tentatively assigned as a *cis*-isomer, *i.e.*, 13-*cis*-3'-alkyl lutein ether. Further characterization of the individual peaks is needed to establish whether the peaks have the epilutein stereochemistry.

When these nine purified lutein ethers were combined in equal molar amounts and run on HPLC, we observe a combined chromatogram with isolated peaks. Although the retention times of these ethers have not been previously reported and can not be compared to authentic standards, the trend of increasing T_R is observed for the series as anticipated. This wide range of observed retention times provides us a series of carotenoids suitable for use as internal standards in the quantitative analysis of carotenoids in human serum and other biological samples with the option to select a standard that is non-interfering. It is apparent that peak areas obtained from equimolar amounts of the different alkyl ethers are not identical and that an appropriate conversion factor will be required for accurate quantitation using these standards.

Chapter IV Conclusions

4.1 Oxidation of Zeaxanthin

Three peaks (p2, p3, p4) were isolated in sequence on HPLC from the zeaxanthin oxidation reaction mixture. HPLC, UV/Vis and MS information show they are all rhodoxanthin. The reaction produced these isomers in a net yield of 48% based on starting zeaxanthin. They are tentatively identified here as the three C6 and C6' geometrical isomers (Fig.2.1) of rhodoxanthin. p2 is observed to be a mixture of rhodoxanthin isomers, separable by normal phase HPLC, having additional geometrical isomerism within the polyene chain; p3 is a pure compound and identified as a rhodoxanthin cis-isomer (6-cis or 6,6'-di-cis, Fig.2.1); p4 appears to be all-trans rhodoxanthin. Rhodoxanthin C6 and C6' geometrical isomers have been previously reported³⁹ but we are the first to observe the additional cis/trans isomers involving the polyene chain. 2 mg of authentic all-trans rhodoxanthin was obtained as a gift from Hoffmann-La Roche, but this sample proved to be too old and was a mixture of components as shown by HPLC and NMR, so the co-injection with authentic sample was not possible. We conclude that all-trans rhodoxanthin is a labile compound and great care should be taken in handling and storage. Lack of suitable NMR data prevents unambiguous identification of these cis isomers. Future work should focus on more efficient isolation methods to obtain larger samples needed for NMR and fuller characterization.

4.2 3'-Alkyl Lutein Ethers

Reaction of lutein with alcohols under acidic conditions results in the formation of products that have longer retention times on reversed phase HPLC than lutein, consistent with a less polar structure. Two components were obtained from the reaction mixture by separation using HPLC. UV/Vis shows a weak peak at 331 nm in the spectrum of the second component which is consistent with a *cis* structure. Mass Spectra are completely consistent with assignment of these two components as 3'-alkyl lutein ethers. The parent ion of each ether is observed in the MS and the characteristic loss of ROH from the $[M+H]^+$ ion is observed for all of the ethers isolated. The observation that the dominant fragment in MS is $[MH-ROH]^+$ is consistent with the assignment that the ether is formed at the C3' allylic site of the epsilon ring. Based on the above information, the first component was identified as a mixture of 3'R and 3'S-alkyl lutein ether diastereoisomers, and the second one was tentatively assigned as a *cis*-isomer, *i.e.*, 13-*cis*-3'-alkyl lutein ether. Quantities necessary for NMR have not been isolated in this study.

These alkyl lutein ethers offer considerable utility as internal standards in the investigation of carotenoids using HPLC. It is possible to select a particular derivative with a retention time suitable to avoid coelution with analytes in most samples regardless of the composition. Furthermore, the chemical similarity to the carotenoids of interest guarantees that factors which contribute to the degradation of the carotenoids of interest will likewise affect the internal standard lutein ether. This latter is especially useful since the anticipated recovery of the lutein internal standard can be determined separately from the analytical sample thereby helping to establish the efficiency of recovery and degradation in the workup of carotenoid samples.

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