INVESTIGATION OF CAROTENOID CONTENTS OF VARIOUS MICROALGAE BY CHROMATOGRAPHIC/SPECTROSCOPIC METHODS

A Thesis Submitted to
the Graduate School of Engineering and Sciences of
İzmir Institute of Technology
in Partial Fulfillment of the Requirements for the Degree of

DOCTOR OF PHILOSOPHY

in Chemistry

by
Ayşegül ERDOĞAN

May 2013
İZMİR

We approve the thesis of **Ayşegül ERDOĞAN**

Examining Committee Members:

Prof. Dr. Ahmet E. EROĞLU
Department of Chemistry, İzmir Institute of Technology

Prof. Dr. Zekerya DURSUN
Department of Chemistry, Ege University

Prof. Dr. Oğuz BAYRAKTAR
Department of Chemical Engineering, İzmir Institute of Technology

Assist. Prof. Dr. Hasan ERTAŞ
Department of Chemistry, Ege University

Assoc. Prof. Dr. Ali ÇAĞIR
Department of Chemistry, İzmir Institute of Technology

31 May 2013

Prof. Dr. Ahmet E. EROĞLU
Supervisor, Department of Chemistry
İzmir Institute of Technology

Prof. Dr. Meltem CONK DALAY
Co-Supervisor, Department of Bioengineering
Ege University

Prof. Dr. Durmuş ÖZDEMİR
Head of the Department of Chemistry

Prof. Dr. R. Tuğrul SENGER
Dean of the Graduate School of
Engineering and Sciences

ACKNOWLEDGEMENTS

It would not have been possible to write this doctoral thesis without the help and support of the kind people around me, to only some of whom it is possible to give particular mention here.

First, I would like to express my deepest gratitude to my supervisor, Prof. Dr. Ahmet E. EROĞLU for his supervision and continuous support he provided. He has given the opportunity to do this work and taught to be persistent to accomplish any goal. His words have always inspired me and brought me to a higher level of thinking. What I learn from him is not just to meet the graduation requirement, but how to view this world from a new perspective.

This thesis would have remained a dream had it not been for my co-supervisor, Prof. Dr. Meltem CONK DALAY. I cannot find words to express my gratitude because of her endless help and excellent suggestions to complete my reserach. She always stands by me.

I would like to show my greatest appreciation to Assoc. Prof. Dr. Ali ÇAĞIR. I cannot say thank you enough for his tremendous support and help. I feel motivated and encouraged every time I attend his meeting. Without his encouragement and guidance this project would not have materialized.

I also thank to Assoc. Prof. Dr. Hasan ERTAŞ for his recommendations. He gave me constant motivation and support during the course of my thesis.

Moreover, I am grateful to examining committee members of my thesis, Prof. Dr. Zekerya DURSUN and Prof. Dr. Oğuz BAYRAKTAR for spending their time on careful reading of my thesis as well as for their valuable comments.

A special thanks goes to my team mate, Hayriye Soykan, who helped me to complete this work and gave suggestions about the experiments. Also thanks to Canberk İŞLER and Dr. Zeliha DEMİREL for cultivating the microalgae continously and their significant contribution to this study.

I wish to express my gratitude to my best friends Meral KARACA and Dr. Semira ÜNAL YEŞİLLER who were always willing to help at any time and gave their best suggestions.

Moreover, I am thankful to my colleagues and room mates; Esen DÖNERTAŞ, Merve DEMİRKURT and Yusuf SÜRMELİ for their friendship and inner support.

Besides, I would like to acknowledge The Scientific and Technological Research Council of Turkey for the support of this work through the project TBAG 110T099 for financial assistance and also the Center of Material's Research at IZTECH for their facilities.

I should also thank to my husband, Dr. Murat ERDOĞAN who listened to my ideas and had confidence in me when I doubted myself. He was always there cheering me up and stood by me through the good times and bad.

Last of all, I am greatly indepted to my family. Thanks to my parents Kadir ŞEKER and Mukadder ŞEKER, for giving me life in the first place, for educating me for unconditional support and encouragement to pursue my interests and also to my brother Mehmet Emin ŞEKER, for sharing his experiences with me, for listening to my complaints and frustrations and for believing in me.

For any errors or inadequacies that may remain in this work, of course, the responsibility is entirely my own.

Dedicated to:

"My grandparents: Latif Fuat ÖZTÜRK and Kevser ÖZTÜRK"

ABSTRACT

INVESTIGATION OF CAROTENOID CONTENTS OF VARIOUS MICROALGAE BY CHROMATOGRAPHIC/SPECTROSCOPIC METHODS

Microalgae are the most important energy sources among microorganisms. Carotenoids, as important pigments and antioxidants, are produced by microalgae and are used both for health purposes and as natural colorants. There has been considerable research for the development, identification and determination of new strains of organisms to produce a variety of carotenoids. New methods for the isolation of carotenoids should be developed also for analytical purposes.

This study aimed the biosynthesis of carotenoids from microalgae, (*Prochlorococcus sp.*, *Scenedesmus protuberans* and *Nitzschia sp*.) their identification and quantification. It is known that some types of microalgae can produce high amount of carotenoids under different stress conditions while some others can produce carotenoids only under stress. For this purpose, cultivation conditions were optimized for the production of new or high value of carotenoids in the selected microalgal strains.

Freeze-dried microalgae were extracted using different organic solvents and their carotenoid contents were investigated by high performance liquid chromatography (HPLC) and other chromatographic techniques such as liquid chromatography-mass spectrometry (LC-MS); in addition to (UV-VIS) spectroscopy.

In green microalgae lutein (2.54 mg/g for *Prochlorococcus sp.* and 2.45 mg/g for *Scenedesmus protuberans)* is the most abundant carotenoid. On the other hand, in brown microalga fucoxanthin (6.58 mg/g for *Nitzschia sp*.) is the highly accumulated carotenoid. Under stress conditions, many microalgae alter their biosynthetic pathways for the formation and accumulation of carotenoids. Therefore, the effect of different nitrogen sources, oxidative stress conditions and different light sources on lutein content in green microalgae and on fucoxanthin content in brown microalga were investigated.

ÖZET

ÇEŞİTLİ MİKROALGLERİN KAROTENOİD İÇERİKLERİNİN KROMATOGRAFİK/SPEKTROSKOPİK YÖNTEMLERLE ARAŞTIRILMASI

Mikroskobik canlılardan mikroalgler önemli enerji kaynaklarından biridir. Önemli pigment ve antioksidan kaynağı olan karotenoidler mikroalgler tarafından üretilen ürünlerdendir; sağlık amaçlı ve doğal renklendirici olarak kullanılırlar. Çeşitli karotenoidlerin üretilmesi için yeni tür organizmaların bulunması özel bir önem kazanmıştır. Karotenoidlerin analitik olarak kullanılması izolasyonuna yönelik yöntemlerin geliştirilmesini de gerekli kılar.

Bu çalışmada, karotenoidlerin mikroalgler tarafından biyolojik yollarla üretimi, ürünlerin karakterizasyonu ve derişimlerinin belirlenmesi hedeflenmiştir. Bu amaçla, mikroalglerden *(Prochlorococcus sp., Scenedesmus protuberans* and *Nitzschia sp*.) karotenoidlerin biyosentezi ile elde edilen karotenoidlerin içerik ve seviyeleri belirlenmiştir. Bazı mikroalglerin farklı stres koşullarında daha fazla karotenoid ürettikleri bilinmektedir. Bazı karotenoidler ise sadece stres koşullarında üretilmektedir. Dolayısıyla, seçilen mikroalglerden yüksek miktarlarda veya yeni karotenoidlerin üretilebilmesi için kültür koşulları optimize edilmiştir.

Liyofilize edilen mikrolagler çeşitli organik çözgenlerle ekstrakte edilmiş ve karotenoid içerikleri yüksek basınçlı sıvı kromatografisi (HPLC), sıvı kromatografi-kütle spektrometri (LC-MS) gibi diğer kromatografik teknikler ve spectroskopi (UV-VIS) ile belirlenmiştir.

Çalışmada kullanılan yeşil mikroalglerde (*Prochlorococcus sp.* için 2.54 mg/g ve *Scenedesmus protuberans* için 2.45 mg/g*)* yüksek miktarda lutein olduğu görülmüş; diğer taraftan kahverengi (*Nitzschia sp.*) mikroalgte ise derişik miktarda fukoksantin (6.58 mg/g) bulunmuştur. Çoğu mikroalg türü stress koşullarında biyosentez mekanizmalarını değiştirmektedir. Bu nedenle, farklı azot kaynakları, oksidatif stres koşulları ve farklı ışık kaynaklarının yeşil mikroalglerde bulunan lutein miktarına; kahverengi algte ise bu koşulların fukoksantin miktarına olan etkisi araştırılmıştır.

TABLE OF CONTENTS

LIST OF FIGURES

Figure **Page**

LIST OF TABLES

CHAPTER 1

INTRODUCTION

Carotenoids are compounds constituted by eight isoprenoid units (ip). The ip units are joined in a head-to-tail pattern, but the order is inverted at the molecule center (Figure 1.1). According to this structure, a numbering system (semi-systematic) was assigned to name carotenoids. Lycopene ($C_{40}H_{56}$) is considered the first colored carotenoid in the biosynthesis of many other natural carotenoids and it is linear. Moreover, it is also common to find acyclic, cyclic, and shortened carotenoids, among others. Consequently, carotenoid biosynthesis involves many chemical reactions to attain such diverse structures (Britton 1996, Delgado Vargas 2000).

1.1. Chemistry

Carotenoids are reported to be ubiquitous organic molecules, but they are not produced by the human body or animals. However, it is well known that carotenoids in animal tissues are obtained from the diet. New research has demonstrated that carotenoids may also lend additional health benefits that may possibly reduce the risk of certain types of chronic diseases such as cancer and heart disease (Chaudhry 2003).

Carotenoids are also important natural sources of orange, yellow, and red food coloring for the food and beverage industries (Otles and Atl 1997). They are lipid soluble pigments responsible for many of the brilliant red, orange, and yellow colors in edible fruits (lemons, peaches, apricots, oranges, strawberries, cherries, etc.), vegetables (carrots, tomatoes, etc.), fungi (chanterelles), flowers, and also in birds, insects, crustaceans, and trout (Linden and Lorient 1999, Goodwin 1980). Carotenoids are also present in animal products such as eggs, lobsters, grayfish, and various types of fish (Linden and Lorient 1999). In higher plants, they occur in photosynthetic tissues and chloroplasts where their color is masked by that of the more predominant green chlorophyll.

In general, carotenoids in foods are C_{40} tetraterpenoids comprised of eight C_5 isoprenoid (ip) units (Figure 1.1) whose order is inverted at the molecule center, joined head-to-tail, except at the center where a tail-to-tail linkage reverses the order, resulting

in a symmetrical molecule (Britton 1996, Goodwin 1980). This produces the parent C_{40} carbon skeleton from which all the individual variations are derived (Britton 1995).

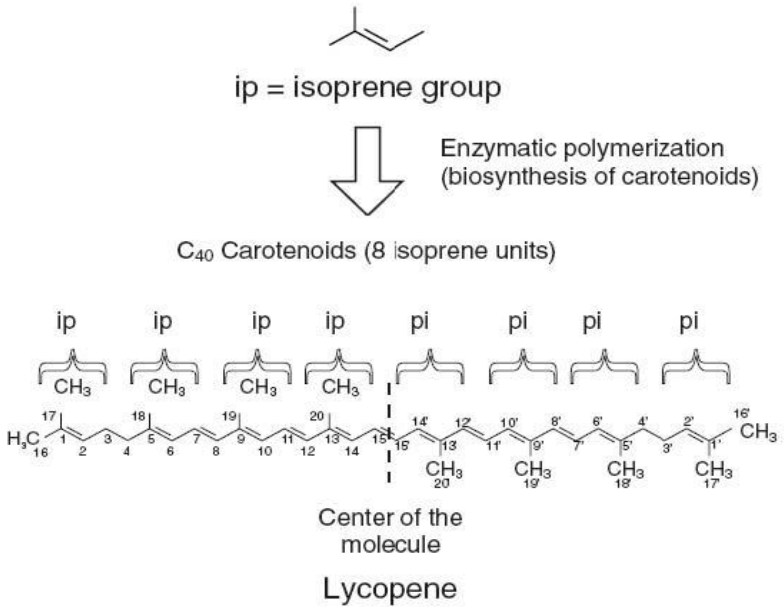


Figure 1.1. Structure of carotenoids and the common numbering system
(Source: DelgadoVargas 2003)

Two main systems have been used to classify carotenoids: (1) by their chemical structure two groups are formed (carotenes and xanthophylls) and (2) by their functionality they are grouped as primary and secondary carotenoids (Table 1.1).

All carotenoids can be considered as lycopene ($C_{40}H_{56}$) derivatives by reactions. This basic skeleton can be modified in many ways including cyclization at one and/or both ends of the molecule to give different end groups, changes in hydrogenation level, dehydrogenation and introduction of oxygen containing functional groups, rearrangement, double bond migration, methyl migration, chain elongation, chain shortening, isomerization, or combinations of these processes resulting in a great diversity of structures (Haila 1999, Rodriguez-Amaya 1997). Figure 1.2. shows some common carotenoid structures.

Table 1.1. Classification of carotenoids
(Source: Delgado-Vargas 2000)

Criteria of classification	Groups and Properties	Examples
By their structural elements	**Carotenes:** Carotenoids constituted by carbon and hydrogen	α-carotene, β-carotene, β-cryptoxanthin
	Xanthophylls: Carotenoids constituted by carbon, hydrogen and additionally oxygen	Lutein, zeaxanthin, violaxanthin, neoxanthin, fucoxanthin
By their functionality	**Primary:** Carotenoids required for photosynthetic process	Lutein, zeaxanthin, violaxanthin, neoxanthin, fucoxanthin, β-carotene
	Secondary: their presence is not directly related with plant survival	α-carotene, capsanthin, lycopene, bixin, astaxanthin,

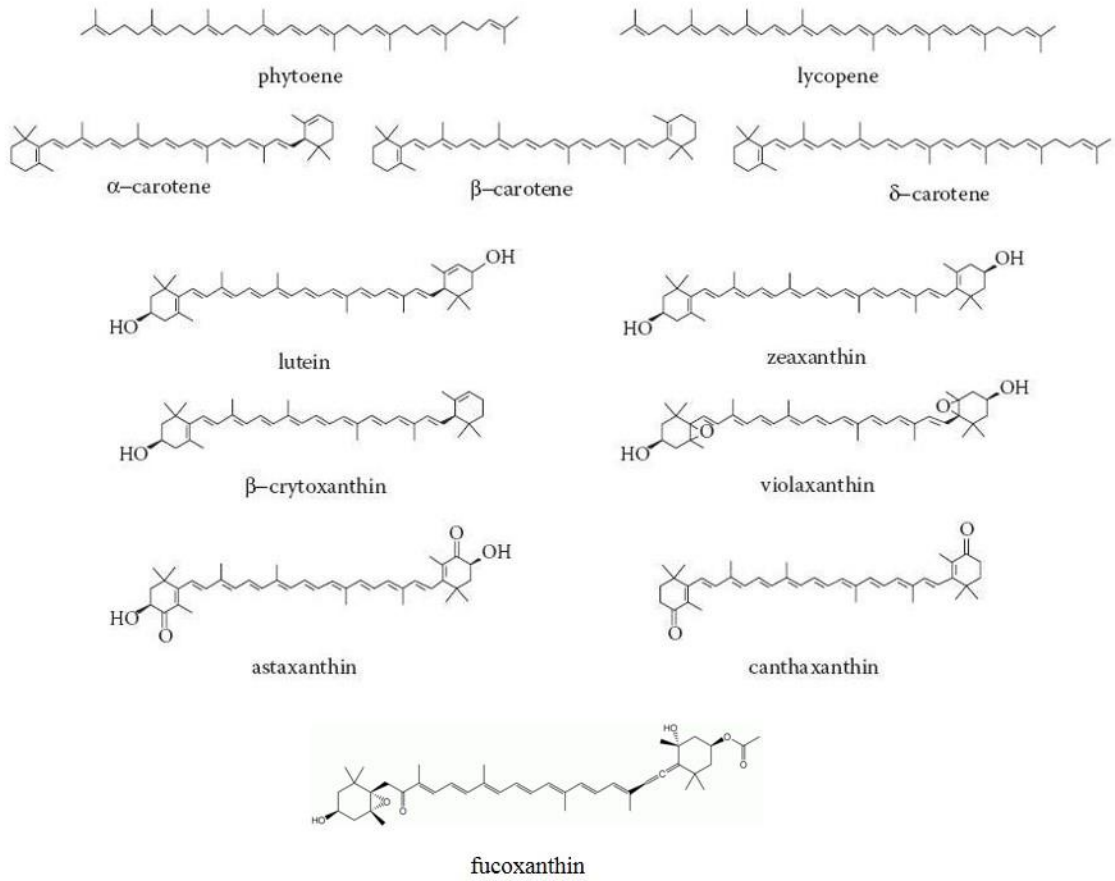


Figure 1.2. Some common carotenoid structures
(Source : Socaciu 2008)

The most characteristic feature of the carotenoid structure is the long system of alternating double and single bonds that forms the central part of the molecule. This constitutes a conjugated system in which the electrons are effectively delocalised over the entire length of the polyene chain. This portion of the molecule (chromophore) is responsible for the molecular shape, chemical reactivity, and light absorption in the visible region of the spectra and hence the colors of carotenoids (Britton 1995, Takyi 2001). At least seven conjugated double bonds are needed for the carotenoids to impart color; phytofluene, with five such bonds, is colorless (Table 1.2). The color deepens as the conjugated system increases, thus lycopene (11 double bonds) is red. Cyclization causes some limitations; hence even though β and α-carotenes have the same number of conjugated double bonds as lycopene, they are orange and orange-red, respectively. The intensity of food color depends on which carotenoids are present, their concentrations, physical states, and the presence or absence of other plant pigments such as chlorophyll (Takyi 2001). Some carotenoid derivatives are associated with beneficial effects on human health. Carotenoids containing retinoid structures (β-ionone rings), such as the α and β-carotenes, serve as precursors of pro-vitamin A. Carotenoids can act as good singlet oxygen quenchers and free radical scavengers due to the many double bonds present in their structures.

Table 1.2. Characteristics of Common Food Carotenes and Xanthophylls
(Source: Takyi 2001)

Name	**Characteristics**
Phytofluene	Acyclic, colorless
Lycopene	Acyclic, red
ζ-Carotene	Acyclic, light yellow
δ-Carotene	Monocyclic (1β ring), red-orange
γ-Carotene	Monocyclic (1β ring), red-orange
β-Carotene	Bicyclic (2β rings), orange
α-Carotene	Bicyclic (1β ring, 1 ε ring), yellow
β-Cryptoxanthin	Bicyclic (2β rings), orange
α-Cryptoxanthin	Bicyclic (1β ring, 1 ε ring), yellow
Zeaxanthin	Bicyclic (2β rings), yellow-orange
Lutein	Bicyclic (1β ring, 1 ε ring), yellow
Violaxanthin	Bicyclic, yellow
Astaxanthin	Bicyclic (2β rings), red

1.2. General Properties of Carotenoids

The carotenoids are lipophilic substances, and thus insoluble in aqueous medium, except in certain cases where highly polar functional groups are present. The presence of the long, extensive system of conjugated double bonds (or polyene chain) is responsible for one of the most distinctive characteristics of the carotenoids, light absorption. A chromophore with seven or more double bonds gives the capacity of absorbing light in the visible range, and consequently, the observation of colors spanning from yellow to red, via a great variety of orange tones. Moreover, the polyene chain makes the carotenoid molecule extremely susceptible to isomerizing and oxidizing conditions such as light, heat, or acids. These properties largely determine the mode of operation and the precautions to be taken when working on the isolation and identification of carotenoids in the laboratory (Britton 1995).

1.2.1. Physical Properties

The physical properties of pure carotenoids, especially their poor stability and low solubility, are particularly significant (Klaui 1981). Carotenoids are unstable in the presence of light and oxygen (Frickel 1984, Dawson and Hobbs 1994, Isler and Gutmann 1971). The central chain of conjugated double bonds is oxidatively cleaved chemically at various points, giving rise to a family of apo-carotenoids. Most carotenoids, but not vitamin A, also serve as singlet oxygen quenchers. Carotenoids can also serve as antioxidants and free radical quenching agents. Carotenoids interact rapidly with free radicals and with oxygen, thereby inhibiting the propagation step of lipid peroxidation.

The chemical structures of carotenoids make them very insoluble in water, but they are fat soluble. With very few exceptions, carotenoids are lipophilic. They are insoluble in water and soluble in organic solvents, such as acetone, alcohol, ethyl ether, chloroform, and ethyl acetate. Carotenes are readily soluble in petroleum ether, hexane, and toluene; xanthophylls dissolve better in methanol and ethanol. Crystalline carotenoids may be difficult to dissolve in the above solvents but do dissolve in benzene and dichloromethane. Carotenoids in the food matrix are relatively stable during typical thermal processing (Nguyen and Schwartz 1998). Several precautions are necessary in handling carotenoids, e.g., carrying out experiments under dim light, evaporation by

rotary evaporator under nitrogen gas flow, storage in the dark under nitrogen or argon at –20°C, and use of antioxidants such as butylated hydroxyanisol, pyrogallol, or ascorbic acid. Because of their highly conjugated doublebond systems, carotenoids show characteristic ultraviolet and visible absorption spectra (Britton 1995b). For most carotenoids, three peaks or two peaks and a shoulder absorb in the range of 400 to 500 nm. Light absorbances of selected carotenoids are shown in Table 1.3. Both the wavelength maximum and are significantly affected by the solvent used.

Table 1.3. Light Absorbance of Selected Carotenoids
(Source: Britton 1995, De Ritter 1981)

Carotenoid	**Solvent**	**Absorption Maximun (nm)**	$E_{1cm}^{1\%}$
Cantaxanthin	Light petroleum	466	2200
α-Carotene	Light petroleum	422, 444, 474	2800
β-Carotene	Light petroleum	425, 453, 479	2592
β-Cryptoxanthin	Light petroleum	425, 452, 479	2386
Lutein	Ethanol	421, 445, 475	2550
Lycopene	Light petroleum	444, 472, 502	3450
Neoaxanthin	Ethanol	416, 439, 467	2243
Violaxanthin	Ethanol	420, 443, 470	2550
Zeaxanthin	Light petroleum	426, 452, 479	2348

1.2.2. Chemical Properties

The fundamental chemistry of carotenoid radicals and the reactions with oxidizing agents, peroxy radicals, etc., is important for evaluating the proposed actions of carotenoids as antioxidants. Oxidation, the major cause of carotenoid loss, depends on available oxygen and the carotenoid involved, and is stimulated by light, heat, peroxides, metals such as iron, and enzymes, while inhibited by antioxidants such as tocopherols and ascorbic acid. Oxidation therefore leads to complete loss of activity while isomerization leads to reduced activity (Takyi 2001).

The overall size and shape of a molecule are extremely important in relation to the properties of a carotenoid and hence to function. All colored carotenoids in the all--trans configuration have extended conjugated double bond systems and are linear, rigid molecules. The cis isomers, however, are no longer simple linear molecules. Their overall shapes differ from those of the all-trans forms, so their ability to fit into subcellular structures may be greatly altered (Britton 1995b). During isomerization, the carotenoid molecules fold back and change from the naturally occurring trans form to the cis form. The conditions necessary for the isomerization and oxidation of carotenoids are likely to exist in home preparations, industrial processing, and during storage of foods. The polyene chain is the cause of the instability of carotenoids, including their susceptibility to oxidation and geometric isomerization. Heat, light, and acids promote isomerization of trans carotenoids, their usual configuration in nature, to the cis-form (Falconer 1964).

1.2.3. Spectroscopic Properties

The characteristic visible light absorption spectrum of the carotenoid pigments is due to the system of conjugated double bonds of their hydrocarbon chain (polyene). For a given carotenoid, the position of the bands of maximum light absorption (λ_{max}) is a function of the number of conjugated double bonds in the molecule (Figure 1.3.).

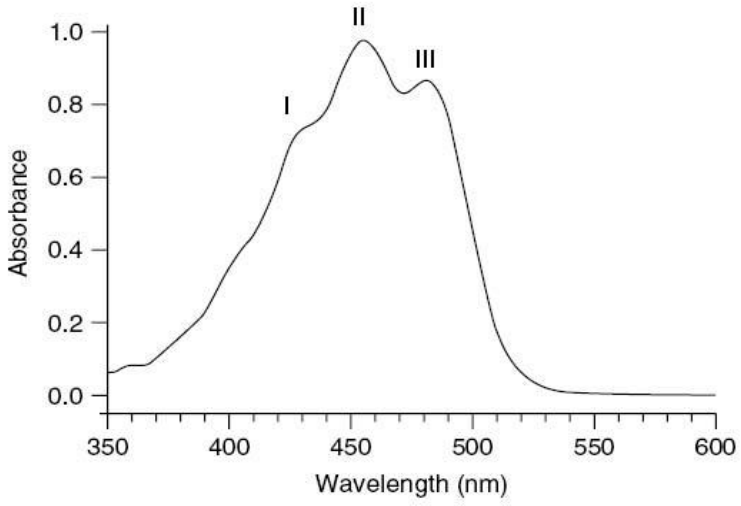


Figure 1.3. General ultraviolet (UV)–visible light absorption spectrum of carotenoids (Source: Hurst 2008)

The absorption maxima are usually referred to using roman numbers (I, II, III). The introduction of a new conjugated double bond into the chromophore causes a bathochromic displacement (to higher wavelength) of 20–22 nm in the absorption maxima (λ_{max}), although this effect may be modified by the presence of different end groups. If the end group is βring, the contribution to the chromophore is only 9–11 nm. In the case of a ε-ring, the conjugation of its double bond is lost and it does not participate in the chromophore. If the double bond of the βring is replaced by a 5,6-epoxide group, there is a hypsochromic displacement of 6–9 nm in λ_{max}. Figure 1.4 shows some of the changes in the electron absorption spectrum depending on the nature of chromophore.

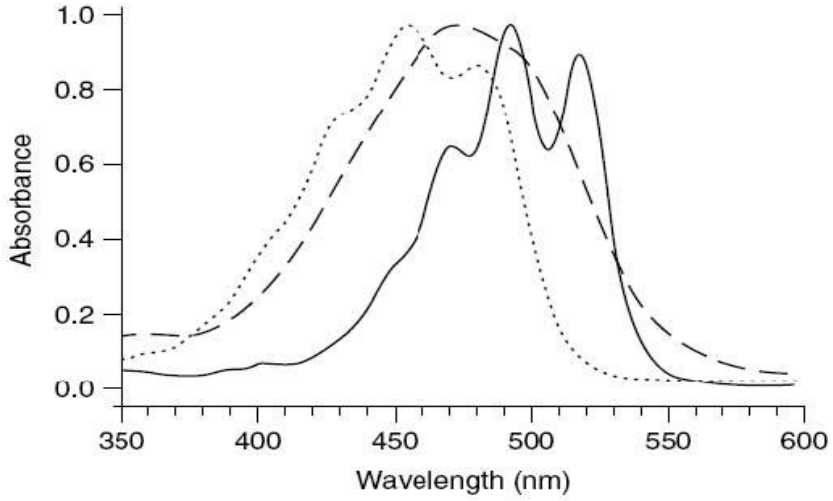


Figure 1.4. Effect of structure and end groups on the spectral fine structure. Lycopene (—), β-carotene (_____), and capsanthin (– –) (Source: Hurst 2008).

The shape of the absorption spectrum of the carotenoid, and the positions of the absorption maxima, can vary depending on the interactions of the molecule with the solvent or lipid environment in which it is dissolved. In general, solvents with low polarity have little effect on the position of the absorption maxima, so that for a given carotenoid, the values of λ_{max} are almost identical in hexane, light petroleum, diethyl ether, methanol, and ethanol. In contrast, very polar solvents such as chloroform, benzene, and pyridine cause very significant bathochromic displacements (10–25 nm), which are extreme in the case of carbon disulfide (30–40 nm). Figure 1.5 compares the fine structure of the ultraviolet (UV)–visible spectra for various carotenoids. When comparing the absorption spectrum of a given carotenoid, it is important to compare not

only the positions of the absorption maxima (λ_{max}), but also the shape and fine structure (defined by percent of III/II). Carotenoid pigments also present other spectroscopic properties such as fluorescence and absorption of energy in the infrared (IR) region. Fluorescence is a property rarely present in the carotenoids, and in fact, only a few carotenoids fluoresce when they are excited at appropriated wavelengths. Therefore, fluorescence spectroscopy is not frequently used in carotenoid studies. Similarly, the use of IR spectroscopy is restricted essentially to the identification functional groups, mainly hydroxyl carbonyl, and allene. The conjugated double bond system constitutes the light-absorbing chromophore that gives carotenoids their attractive color and provides the visible absorption spectrum that serves as a basis for their identification and quantification. The color enables analysts to monitor the different steps of carotenoid analysis. Loss or change of color at any time during the analysis gives an indication of degradation or structural modification. For this reason, the color permits visual monitoring. The ultraviolet and visible spectrum is the first tool for the identification of carotenoids (Britton 1995b).

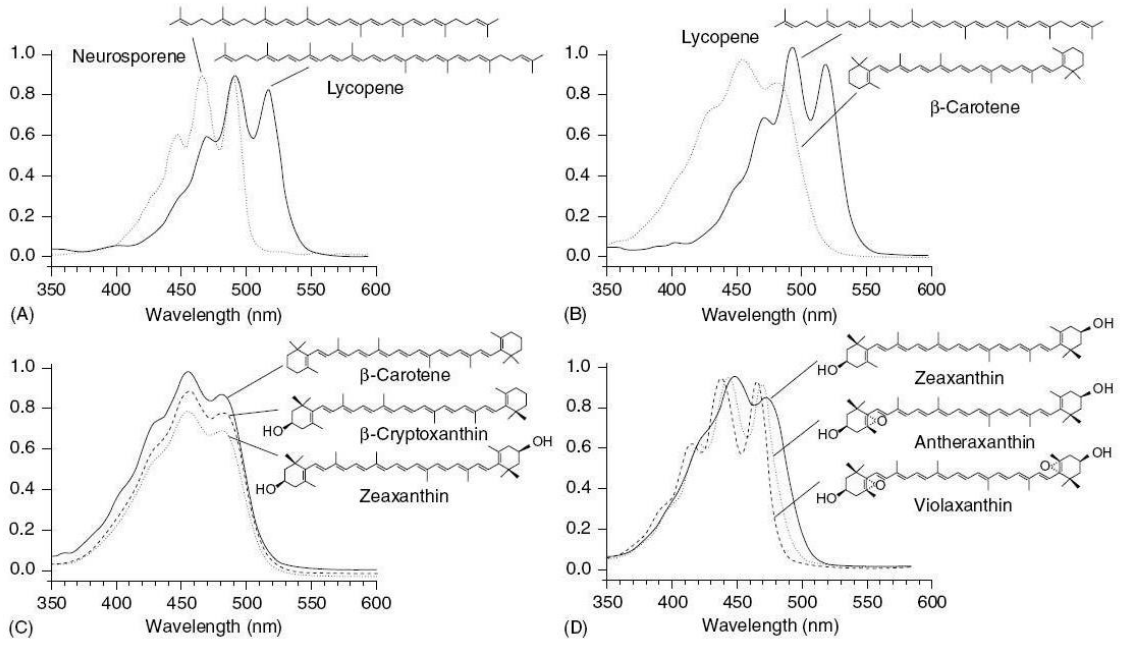


Figure 1.5. Effect of some structural changes of the chromophore on the UV/VIS light absorption spectrum. (A) Increase of chromophore lenght, (B) Cyclization, (C) Hydroxilation, (D) Epoxydation (Source: Hurst 2008).

The wavelength of maximum absorption (λ_{max}) and the shape of the spectrum (spectral fine structure) are characteristic of the chromophore. The λ_{max} values of common carotenoids, taken mainly from Britton's (1995b) compilation, are shown in Table 1.4. Most carotenoids absorb maximally at three wavelengths, resulting in three-peak spectra (Figure 1.6). The greater the number of conjugated double bonds, the higher the λ_{max} values. Thus, the most unsaturated acyclic carotenoid lycopene, with 11 conjugated double bonds, is red and absorbs at the longest wavelengths (λmax at 444, 470, and 502 nm) (Figure 1.6). At least 7 conjugated double bonds are needed for a carotenoid to have perceptible color.

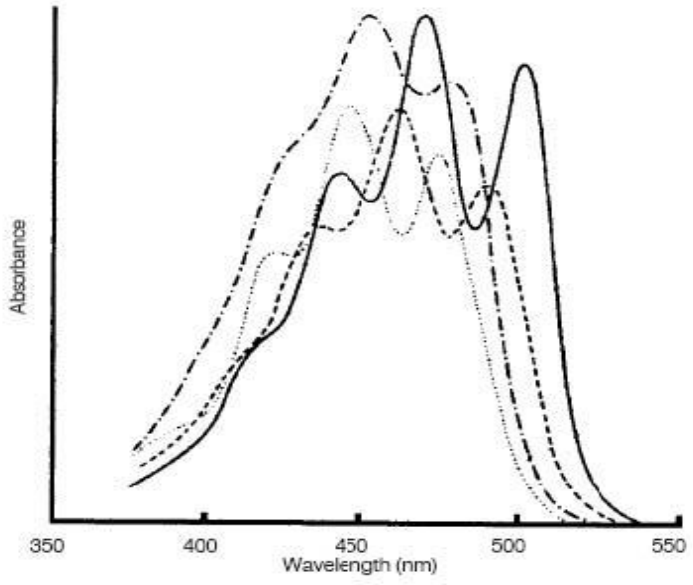


Figure 1.6. Visible absorption spectra of lycopene (__), γ-carotene (- - -), β-carotene (-.-.-.) and α-carotene (....) in petroleum ether (Source: Hurst 2008)

The absorption spectra of carotenoids are solvent dependent. It has to be remembered when spectra are taken by the photodiode array detector in high performance liquid chromatography in which the spectra are taken in mixed solvents in isocratic elution and in varying mixed solvents in gradient elution.

To give an idea of the spectral fine structure, the %III/II can be presented, along with the λ_{max} values. The %III/II is the ratio of the height of the longest wavelength absorption peak, designated III, and that of the middle absorption peak, designated II, taking the minimum between the two peaks as baseline, multiplied by 100 (Britton 1995).

For conjugated keto-carotenoids, such as canthaxanthin and echinenone, the spectrum consists of a broad single maximum, having no defined fine structure, thus %III/II is 0. The absorption coefficient $A_{1cm}^{1\%}$ of a carotenoid (absorbance at a given wavelength of a 1% solution in spectrophotometer cuvette with a 1cm light path) used in the calculation of the concentration also varies pronouncedly in different solvents. Carotenoids in solution obey the Beer -Lambert Law- their absorbance is directly proportional to the concentration. Thus, carotenoids are quantified spectrophotometrically. This quantification, however, depends on the availability of accurate absorption coefficients, which are difficult to obtain (Britton 1995).

1.2.4. Adsorption and Partition Properties

The chromatographic behaviors of carotenoids have a definite relationship with their structures. However, chromatographic data cannot be used as sole criteria for carotenoid's identity. Yet, these data serve as useful complementary information. In normal phase open column chromatography, the adsorption affinity depends on the number of conjugated double bonds, cyclization, and the presence of oxygen substituents.

The influence of the double bonds is best illustrated by the adsorption affinities of the acyclic carotenoids, which elute in the sequence phytoene, phytofluene, ζ-carotene, neurosporene, and lycopene. Comparing monocyclic and bicyclic carotenes, δ-carotene elutes before γ-carotene, and α-carotene elutes before β-carotene. Cyclization decreases the adsorption affinity. Thus, β-carotene is much more weakly adsorbed than γ-carotene, which in turn elutes before lycopene.

The presence of oxygen substituents increases adsorption, the extent of such increase depending on the type, number, and location of the functions. The hydroxyl group exerts a great influence on adsorption; methylation, acetylation, and silylation markedly reduce this effect. The adsorption affinity of a carbonyl group is less than that of a free hydroxyl substituent. The contribution of the functional groups on adsorption affinity increases in the sequence (Davies 1976).

$$\neg OR < \neg C{=}O < 2\,[\neg C{=}O] < \neg OH < \neg COOH$$

In the current widely used reversed phase HPLC, in which partition is the major chromatographic mode, the order is more or less the reverse of that seen in normal phase adsorption open column chromatography. The more polar xanthophylls elute well before the carotenes; the monohydroxy carotenoids elute between these two groups. Elution of carotenes does not always follow the expected pattern and differs with the type of column (monomeric or polymeric) and the mobile phase.

1.2.5. Isomerization and Oxidation

The highly unsaturated carotenoid is open to isomerization and oxidation. Heat, light, acids, and adsorption on an active surface (e.g., alumina) promote isomerization of trans carotenoids, their usual configuration, to the cis forms. This results in some loss of color and provitamin A activity. Oxidative degradation, the principal cause of extensive losses of carotenoids, depends on the availability of oxygen and is stimulated by light, enzymes, metals, and co-oxidation with lipid hydroperoxides. Carotenoids appear to have different susceptibilities to oxidation, ζ-carotene, lutein, and violaxanthin being cited as more labile. Formation of epoxides and apocarotenoids (carotenoids with shortened carbon skeleton) appears to be the initial step (Figure 1.7).

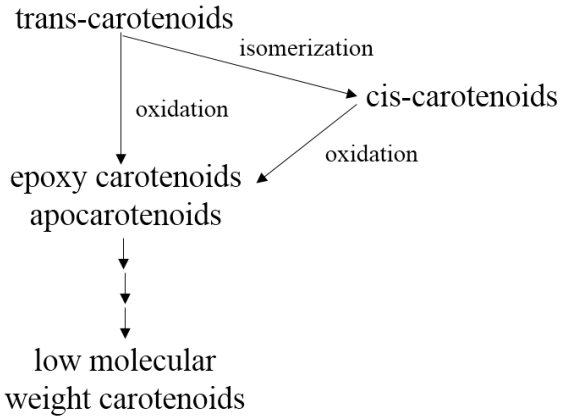


Figure 1.7. Possible scheme for the degradation of caretenoids
(Source: Rodrigez-Amaya 1999a)

Subsequent fragmentations yield a series of low-molecular-weight compounds similar to those produced in fatty acid oxidation. Thus, total loss of color and biologic activities are the final consequences. Conditions necessary for isomerization and

oxidation of carotenoids exist during preparation, processing, and storage of food. Thus, retention of naturally occurring or added carotenoids in prepared, processed, and stored foods is an important consideration. Carotenoids are also subject to isomerization and oxidation during analysis, and preventative measures must be taken to guarantee the reliability of analytic results.

1.2.6. Nutritional and Beneficial Properties

Nutritionally, the main physiological function of the carotenoids is their capacity as precursors of vitamin A, so that they are said to have provitamin A value. This important quality has led some authors to propose their classification according to nutritional activity (depending on their provitamin A character) and their biological activity (antiulcer, anticancer, immunological regulators, antenna photosynthetic pigments, etc.) (Bendich and Olson 1989, Swanson and Parker 1996). The condition for a carotenoid to have such activity is that it possesses at least one unsubstituted end group with a β-ring. β-carotene presents the greatest potential activity, since the central enzymatic cleavage of its molecule originates two molecules of vitamin A (Figure 1.8). Other carotenes such as α-carotene, γ-carotene, β-apo-8'-carotenal, and β-cryptoxanthin give rise to only one molecule of vitamin A, as they possess only one β-ring in their structure.

There are various factors in the effectiveness of the antioxidant action. Among these are the presence of oxygenated functional groups in the structure of the pigment, the conditions of the medium where the pigment acts and the nature of the pro-oxidant substance (Everett 1996). Any of these factors may cause a self-oxidizing effect in place of the expected antioxidant beneficial one. Nevertheless, different in vitro and in vivo studies have concluded that the antioxidant action of pigments such as β-carotene and lycopene is effective.

Other health benefiting effects of the carotenoid pigments derive from their antioxidant action, which can protect against certain cancers and tumors related with the appearance of free radicals (pro-oxidant substances).

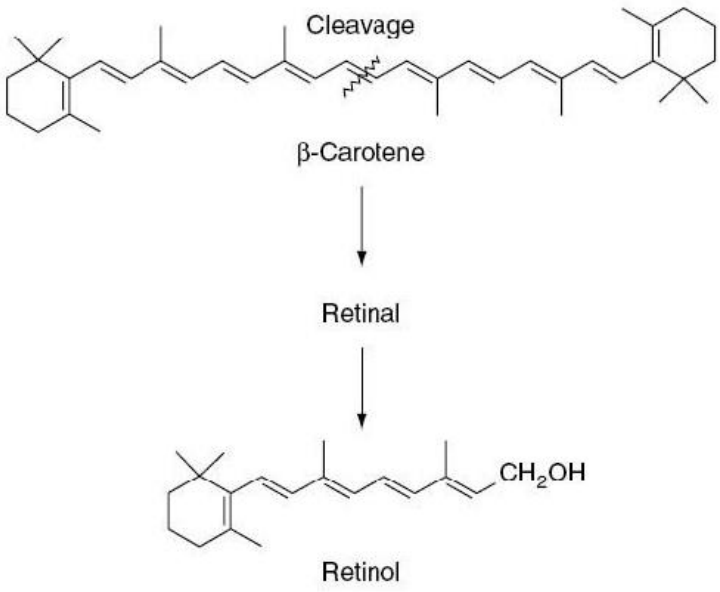


Figure 1.8. Central enzymatic cleavage of β-carotene molecule to give two molecules of Vitamin A (retinol) (Source: Hurst 2008)

By intercepting these harmful substances, the carotenoid pigments become chemi-protectors or anti-cancerigenic substances. The presence of oxygenated functional groups also modifies the bioavailability of these compounds. It has been demonstrated recently that some keto-carotenoids are more rapidly absorbed and metabolized than other carotenes such as, for instance, lycopene. These xanthophylls do not present provitamin A activity, but their antioxidant action is more effective than that of β-carotene. The incorporation of the carotenoid pigments into cell structures is affected by the pigment structure and the presence of functional groups that may modify the interaction with other molecules. Such structure, as mentioned above, determines the effectiveness of the pigment's action.

1.3. General Procedure in Carotenoid Analysis

Carotenoid analysis usually consists of sampling and sample preparation, extraction, saponification, partition to a solvent compatible with the subsequent chromatographic step, and washing, concentration or evaporation of solvent, chromatographic separation, and identification and quantification.

In carotenoid analysis errors can be introduced in each of these steps. Common sources of error in carotenoid analysis are incomplete extraction; physical losses during

the different steps, such as incomplete transfer of carotenoids from one solvent to the other when partition is carried out, loss of carotenoids in the washing water, and partial recovery of carotenoids adhering to walls of containers when carotenoid solution are brought to dryness; incomplete chromatographic separation; misidentification; faulty quantification and calculation; and isomerization and oxidation of carotenoids during analysis or storage of food samples before analysis. A good understanding of the purpose of each step and the possible errors is therefore required. Because of the various factors that affect the carotenoid composition, proper sampling and sample preparation to obtain representative and homogeneous samples for analysis are of importance. Therefore, laboratory work should be planned so that the samples are analyzed as soon as they are collected because it is difficult to store samples without changing their carotenoid composition, even at very low temperature. Because carotenoid concentration is expressed per unit weight of sample, changes in the sample's weight during storage also affect the final result (Rodriguez-Amaya 1999a).

1.3.1. Sample Preparation

The sample that is brought to the laboratory is usually too large, both in bulk and in particle size, for direct analysis. It must therefore be transformed into a homogenous, small sample for analysis, while maintaining its representativity. The problems encountered by analysts in the preparation of samples for analysis include; difficulty in obtaining representative small samples from large samples, loss of material, difficulty in removal of extraneous material from samples without removal of sample constituents including the analyte, enzymatic changes before and during analysis, compositional changes during grinding and changes in unstable components.

The sample preparation procedure should be adapted to the nature of the sample, the analyte, and the analytical method, as well as to the distribution of the analyte in the sample. If the analysis is not going to be performed immediately, the sample should be stored in the refrigerator. In samples with a high content in water, lyophilization may be useful to remove the water (Pomeranz and Meloan 1994).

1.3.2. Extraction

Extraction procedure is one of the most critical step in carotenoid analysis because a good extraction procedure should release all the carotenoids from the food matrix and bring them into solution, without altering them. Since carotenoids are found in a variety of foods, bacteria, algae etc. the extraction procedure should be adapted to suit the sample being analyzed.

The solvent chosen should efficiently extract all carotenoids present in the sample. Because the solvents used in extraction or partition will be removed or at least reduced by evaporation, solvents with low boiling points should be chosen to avoid prolonged heating. When extracting carotenoids from biological samples, which contain large amounts of water, a water-miscible organic solvent (e.g., acetone, methanol, ethanol, or mixtures thereof) should be used to allow better solvent penetration. Dried materials can be extracted with water-immiscible solvents, but extraction is usually more efficient if the samples are rehydrated first and then extracted with water-miscible solvents. Acetone has been widely used in carotenoid extraction; however, the advent of high performance liquid chromatography (HPLC) has seen tetrahydrofuran (THF) become a popular extraction solvent. $MgCO_3$ or another neutralizing agent is often added to neutralize the acids liberated extraction in order to prevent isomerization and degradation. While performing extraction it is recommended to keep it as short as possible not only to prevent enzymatic oxidation, but also make the addition of neutralizing agents unnecessary (Britton 1995a).

1.3.3. Saponification

Another important process in the study and processing of carotenoids is the saponification. Saponification is an effective means of removing chlorophylls and unwanted lipids, which may interfere with the chromatographic separation and shorten the column's life. In samples like fruits, saponification hydrolyzes the carotenol esters. This simplifies the chromatographic separation, identification, and quantification because the free carotenols are analyzed instead of the carotenol esters, which are usually present in a difficult toseparate mixture of esters with a variety of fatty acids. Saponification and the subsequent washing, however, can result in losses of carotenoids, especially xanthophylls. Hence it should be omitted from the analytical

procedure whenever possible. When indispensable, saponification is best carried out by adding 10% methanolic KOH. (However this amount depends on the type of the sample to be analyzed and must be optimized). The resulting mixture is left overnight at room temperature in the dark, and then the carotenoid solution is washed five times with water to remove the alkali.

Carotenoids appear in nature as mixtures of carotenes and xanthophylls with fatty acids; some of these xanthophylls have hydroxyl groups and they are found as a mixture of fatty acid esters. Consequently, saponification is important to obtain a less complex mixture for analysis because the same hydroxyl-carotenoid appears as more than one compound by the esterification level and/or the fatty acids involved, and this process eliminates chlorophyll. Alkaline treatment is the most common saponification procedure but some carotenoids are highly sensitive (e.g., the keto-carotenoids such as astaxanthin and fucoxanthin) and, therefore, the use of microbial lipases has been proposed. Candida cylindracea lipase has been utilized for red palm oil saponification and the process is carried out in darkness, under nitrogen atmosphere (Britton 1995a).

1.3.4. Partition

The extract usually contains a high amount of water, which can be removed by partitioning to hexane, petroleum ether, diethyl ether, or dichloromethane, or mixtures of these solvents. Diethyl ether or a mixture of this solvent with hexane or petroleum ether is preferred for extracts with large amounts of xanthophylls, part of which is lost during partitioning with pure hexane or petroleum ether. Partitioning is a part of open column methods, so that chromatography can be started at a low mobile phase polarity, which is then increased during the separation process.

In HPLC methods, the extract is sometimes directly evaporated to dryness and then dissolved in the mobile phase or a solvent compatible with the mobile phase. Partitioning is best done by adding small portions of the acetone, methanol, or THF extract to petroleum ether or another appropriate solvent in a separatory funnel. After the addition of each portion, water is added gently to avoid formation of an emulsion, preferably by allowing it to flow along the walls of the funnel. The two layers are allowed to separate, without agitation, and the lower aqueous phase is discarded.

When the entire extract has been added, the petroleum ether phase is washed

four or five times with water to remove residual acetone, methanol, or THF. To follow the chromatography rule that the sample be introduced into the chromatographic system in the smallest volume possible, the carotenoid solution, after partitioning for unsaponified samples and after washing for saponified samples, is dried with anhydrous sodium sulfate and then concentrated for open column chromatography (OCC) or evaporated to dryness to be taken up in the mobile phase or another appropriate solvent for HPLC.

1.3.5. Chromatographic Separation

The extent to which chromatographic separation is carried out in carotenoid analysis depends on the information desired. Food and algal samples typically contain both the apolar carotenes and the more polar xanthophylls. Whatever the method used, the chromatographic process should be able to cope with this polarity range. Chromatography in descending, gravity-flow (often with slight pressure provided by a water aspirator) columns, currently referred to as OCC, is the classical method of separating carotenoids for quantitative analysis. It is also useful in separating and purifying carotenoids to be used as standards for HPLC. Separation of the carotenoid pigments is followed visually. Low pressure may also be applied at the top of the column (e.g., with nitrogen gas).

Thin-layer chromatography, although efficient in monitoring the progress of chemical tests for identification purposes, is not adequate for quantitative analysis because of the danger of degradation and isomerization on a highly exposed plate. Carotenoids are particularly prone to oxidation by air when adsorbed on the thin layer. Additionally, it is not easy to quantitatively apply the sample on the plate and quantitatively recover the separated carotenoids from the plate for measurement. Gas chromatography is also inappropriate because of the thermal lability and low volatility of carotenoids. A major advantage of OCC is the simple and inexpensive column (i.e., glass column packed with the adsorbent). However, reproducibility and efficiency of the separation of carotenoids depend on the skill, patience, and experience of the analyst, particularly in packing the column and adjusting the volumes and proportions of the eluting solvent, as well as the analyst's acuity for detecting the separation.

The possibility of degradation varies with different stationary phases (adsorbents) and increases as the chromatographic process is prolonged.

Re-chromatography of an impure fraction may sometimes be necessary, extending the analysis time and increasing the danger of carotenoid decomposition.

In OCC, a column has to be packed for each analysis. A definite improvement in HPLC is the possibility of reproducible separations with a reusable column, under controlled conditions, without undue exposure to air or light. Reversed-phase HPLC on C_{18} columns is clearly the preferred mode. Reasons for the popularity of the C_{18} column are its weak hydrophobic interactions with the analytes (thus it is expected to be less destructive than the polar forces in normal-phase OCC), compatibility with most carotenoid solvents and the polarity range of carotenoids, and wide commercial availability. Many different C_{18} reversed-phase materials are available from different manufacturers and vary in the degree of carbon loading, end capping, and the nature of the bonded phase (i.e., monomeric or polymeric). Lack of reproducibility is a persisting problem. The properties and quality of the same kind of column differ considerably between brands, between batches of the same brand, and even within the same batch (Pfander and Riesen 1994). Thus, some adjustments are often needed when published methods are adapted.

Most carotenoid separations have been carried out with 5-μm C_{18} spherical particles packed in a 250 × 4.6 mm column. Some laboratories are already using shorter and narrower (narrow bore) columns, smaller particles, and a C_{30} stationary phase. Most commercial reversed-phase columns are now end capped to minimize polar interaction of the silanol residues with the analytes and thus diminish tailing and improve column reproducibility. Monomeric phases are simpler to use and more reproducible. Polymeric C_{18} phases, on the other hand, have been found to have excellent selectivity for structurally similar carotenoids, as in the difficult separation of geometric isomers (Craft , 1990, Lesellier 1989, Quackenbush and Smallidge 1986, Bushway 1985).

Guard columns, which should be changed frequently, are needed for food samples to prevent particulate material and impurities from entering the analytic column, thus prolonging the column's life. It can, however, increase band broadening, and the possibility that part of the carotenoid can be retained in it cannot be overlooked. Metal surfaces, particularly stainless steel frits in the guard and analytic columns, were reported to be damaging to carotenoids (Scott 1992). Thus, the use of metal-free columns (Craft 1992) and poly ether ether ketone (PEEK) tubing for column connections (Hart and Scott 1995) has been recommended.

The most important properties to be considered in selecting the mobile phase are polarity, viscosity, volatility, and toxicity. In addition, it must be inert with respect to the carotenoids. Many solvent systems have been suggested as mobile phases for carotenoids, but the primary solvents are acetonitrile and methanol, and most systems are actually slight modifications of some basic combinations (Craft 1992). Acetonitrile has been widely used because of its lower viscosity and slightly better selectivity for xanthophylls when monomeric C_{18} column is used (Khachik , 1986). Epler , (1992) reported, however, that with methanol-based solvents, higher recoveries of carotenoids occurred in almost all of 65 columns tested. Methanol is also more available, less expensive, and less toxic than acetonitrile. Recovery of carotenoids from the column was improved when ammonium acetate was added to acetonitrile-based solvents. Addition of triethylamine to the mobile phase containing ammonium acetate further increased recovery, from around 60% to over 90% (Hart and Scott 1995).

Small amounts of other solvents are added to obtain the desired retention, increase solubility, and improve resolution. Frequently used for this purpose are chlorinated solvents (e.g., chloroform and dichloromethane) because of their good solvent properties and effects on selectivity, although these solvents can be contaminated with traces of hydrochloric acid. Other solvents used as modifiers are tetrahydrofuran, ethyl acetate, hexane, acetone, and water. Some methanol has also been added to acetonitrile-based mobile phase. Craft , (1992) investigated nine solvent modifiers and found tetrahydrofuran to be the most beneficial modifier of methanol. There is a tendency to use mixtures of three or more solvents. Many workers, however, use solvent mixtures containing water.

Gradient elution should only be used when the analysis cannot be done isocratically. Isocratic separation is rapid, can be performed with simple equipment (with a single high-pressure pump and premixed solvent), and results in stable baseline and more reproducible retention times. It is usually sufficient for the determination of provitamin A carotenoids or the principal carotenoids of food samples. Gradient elution has the advantage of greater resolving power, improved sensitivity, and elution of strongly retained compounds. It is more likely to resolve the whole range of carotenoids found in a given food. However, it has several disadvantages: 1) increased complexity, 2) requirement for more sophisticated and expensive equipment, 3) need for column re-equilibration between runs, 4) greater differential detector's response (i.e., different detector's signals for the same concentration of different compounds), and 5) often poor

reproducibility. The column must be brought back to the starting solvent and equilibrated for 10–30 minutes in this solvent before a new run is commenced. Good solvent miscibility is required to prevent baseline disturbance resulting from outgassing and refractive index effects (Craft 1992). Because of the qualitative and quantitative variation of the carotenoid composition of foods, it is doubtful that a single chromatographic system can be established that can be applicable to the different foods.

At least some modification of the mobile phase is needed when changing from one food to another. The injection solvent must be compatible with the HPLC mobile phase. If the carotenoids are much more soluble in the injection solvent than in the mobile phase, and especially if the solution is nearly saturated, the carotenoids will precipitate on injection, leading to peak tailing, or they will remain in the injection solvent while passing though the column, resulting in broad bands and doubled peaks (Craft 1992). On the other hand, the sample will not dissolve completely if the solvent is too weak. Samples can be injected in the mobile phase to avoid this problem of incompatibility. However, because of the solubility range of carotenoids in food samples, another solvent may be preferred for solubilization and injection. Porsch (1993) suggested that sample solvent– mobile phase viscosity should be kept fairly below 2, and the much higher dissolving power of the injection solvent should be decreased by mixing with the mobile phase before injection.

1.3.6. Identification and Quantification

The chromatographic behavior and the ultraviolet visible absorption spectrum provide the first clues for the identification of carotenoids. Both the position of the absorption maxima (λ_{max}) and the shape (fine structure) of the spectrum reflect the chromophore. In HPLC the availability of the photodiode array detector allows the acquisition of the spectra online, making the use of this criterion easier. Spectra can be taken, stored, and subsequently compared with those of standards.

Identification of carotenoids based solely on the retention times and the absorption spectra may lead to wrong conclusions. Retention times are difficult to reproduce even within a laboratory and may vary during the course of a day. Even when carotenoid standards are available and spiking can be done, identification is still not conclusive because different carotenoids can have the same retention time in a given chromatographic system. In addition, different carotenoids may have the same

chromophore, thus presenting the same absorption spectrum. Because of the widespread use of these two parameters as the only criteria, cases of misidentification can be discerned in the literature.

Thus, in literature it is recommended that the following minimum criteria be fulfilled for identification (Schiedt and Liaaen-Jensen 1995, Pfander 1994):

1) the visible (or ultraviolet for shorter chromophores) absorption spectrum (λ_{max} and fine structure) in at least two different solvents must be in agreement with the chromophore suggested;
2) chromatographic properties must be identical in two systems, preferably TLC (RF) and HPLC (tR), and chromatography with an authentic sample should be demonstrated; and
3) a mass spectrum should be obtained, which allows at least confirmation of the molecular mass. The requirement of a mass spectrum, however, would limit carotenoid analysis to a very few laboratories around the world, although it is very much needed.

Moreover, common, major carotenoids can be conclusively identified by the combined use of chromatographic data, absorption spectra, and specific chemical reactions, the latter to confirm the type, location, and number of functional groups.

Mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy are, however, indispensable in the elucidation of unknown or inconclusive structures of carotenoids. Especially, MS has been extensively used to elucidate the structures of carotenoids in algae, fungi, and bacteria in recent literatures.

In addition to identification, the quantification step in OCC methods is fairly straightforward. The separated carotenoid fractions are collected and quantified spectrophotometrically through the use of tabulated absorption coefficients. The absorption coefficient $A^{1\%}$ 1cm of a carotenoid (absorbance at a given wavelength of a 1% solution in 1 cm light path spectrophotometer cuvette), used in the calculation of the concentration. In quantitative analysis by HPLC, the following facts should be considered: carotenoids absorb maximally at different wavelengths and have different absorption coefficients; solvent effects on absorption are substantial (tabulated absorption coefficients and λmax values refer to single solvents, mobile phase in HPLC isocratic elution is usually a mixture, and in gradient elution the mixture's composition varies during the chromatographic process); and obtaining and maintaining carotenoid standards, which are required for calibration, are difficult.

Modern liquid chromatographs allow measurement of carotenoids at the wavelengths of maximum absorption. HPLC quantification is carried out by means of internal or external calibration for which the concentrations of the standards are also determined spectrophotometrically as in OCC.

A constant supply of carotenoid standards is needed in HPLC methods, especially when external standardization is used. The accuracy of the analytic results depends on how accurately the concentrations of the standard solutions are known. Unfortunately, only a few carotenoid standards (e.g., α-carotene, β-carotene, and lycopene) are available commercially. Other carotenoids have to be isolated and purified from natural sources by the analyst. This can be done by OCC or by accumulating separated fractions from several HPLC runs. Both procedures are time consuming and tedious and require experience and patience. An ideal commercially available internal standard has not been encountered. It is not easy to find a readily available and stable compound that has chemical and spectral properties similar to those of the carotenoids. Synthetic carotenoids, such as β-apocarotenal, canthaxanthin, and echinenone, which are not found in the samples being analyzed, have also been used but are subject to instability problems as the sample's carotenoids. The instability of carotenoid standards is a serious problem. Standard carotenoid crystals should be sealed in ampoules under nitrogen or argon and stored at –20°C or preferably at –70°C until use. Stock and working solutions, even when kept at low temperature, have limited validity; the analyst should know when degradation commences under his laboratory's conditions. The analyst has to prepare solutions of various concentrations, inject each of these solutions, and construct the curve. Inaccuracies in the preparation of the solutions, determination of the concentrations, and construction of the calibrating curves will affect the reliability of the results. In both OCC and HPLC, accurate quantification requires conclusive identification and optimal separation of the carotenoids.

It is obvious that in quantitative HPLC analysis the accuracy is dictated by how accurately the peak areas are determined. The continued improvement in column efficiency resulting in chromatograms with well resolved peaks is reassuring, indicating that this source of error is ceasing to be a serious problem in carotenoid analysis. Aside from the internal standard for calibration, standards also termed internal standards have been added at the beginning of the analysis to investigate losses of carotenoids during extraction and the entire workup procedures.

1.4. Sources of Errors in Carotenoid Analysis

It is known that carotenoid analysis is difficult due to several factors (Rodriguez-Amaya 1999a). First of all, there are many naturally occurring carotenoids. Thus, identification, and accurate quantification, is not easily accomplished. The limited commercial availability of carotenoid standards is also a serious problem to carotenoid analysis. In addition, the carotenoid composition of foods or algae differ qualitatively and quantitatively. Therefore, the analytical procedure, principally the chromatographic steps, has to be adapted to the carotenoid composition of each sample. Since the nature of the matrix differ, sample preparation, extraction, and storage conditions should be established for each sample to be analyzed. On the other hand, the highly unsaturated carotenoid molecule is susceptible to isomerization and oxidation, which can easily occur during analysis and/or during storage of samples prior to analysis.

1.4.1. Common Errors in Carotenoid Analysis

In carotenoid analysis, errors can be introduced in each step of the analytical procedure. Common sources of error in carotenoid analysis are:

- samples not representing the food lots under investigation
- incomplete extraction
- physical losses during the different steps, such as incomplete transfer of carotenoids from one solvent to the other during partitioning or loss of carotenoids in the washing water, partial recovery of carotenoids adhering to container walls when carotenoid solutions are evaporated to dryness
- incomplete chromatographic separation
- erroneous identification
- faulty quantification and calculation
- isomerization and oxidation of carotenoids during analysis and/or during storage of food samples before analysis.
- saponification

Due to these various factors that affect the carotenoid composition of samples, laboratory work should be planned previously so that the samples are analyzed soon after collection because it is difficult to avoid changes in carotenoid composition during sample storage, even at very low temperature. Because carotenoid concentration is

expressed per unit weight of sample, changes in the food's weight during storage also affect the final result.

When storage is unavoidable, samples should be stored at –20ºC (or even lower temperatures for longer periods) and tissue disintegration should be postponed until after storage and then carried out immediately before or simultaneously with extraction. Lyophilization is widely considered the appropriate way to preserve biological samples that have to be stored before carotenoid analysis. Moreover, the instability of carotenoid standards is a serious problem. Standard carotenoid crystals should be sealed in ampoules under N_2 or argon and stored at –20ºC, or better at -70ºC, until use. Stock and working solutions, even when kept at low temperature, have limited validity; the analyst should know when degradation commences under the conditions of his/her laboratory. Although HPLC is currently the preferred method for carotenoid analysis, it is subject to several sources of errors (Kimura and Rodriguez Amaya 1999):

a. incompatibility of the injection solvent and the mobile phase
b. erroneous identification
c. quantification of highly overlapping peaks
d. low and variable recovery of the carotenoids from the HPLC column
e. errors in the preparation of standard solutions and in the calibration procedure and
f. erroneous calculations.

1.4.2. Special Precautions in Carotenoid Analysis

The main problem in the analysis of carotenoids derives from their instability. Thus, whatever the analytical method chosen, several precautions should be taken in order to avoid quantitative losses. These include (Davies 1976, Britton 1991, Schiedt and LiaaenJensen 1995):

1. completion of the analysis within the shortest possible time
2. exclusion of oxygen
3. protection from light
4. avoiding high temperatures
5. avoiding contact with acid
6. use of high purity solvents, free from harmful impurities.

Oxygen, especially in combination with light and heat, is highly destructive. Therefore, oxygen can be excluded at several steps during analysis and during storage through the use of vacuum and a N_2 or argon atmosphere. Antioxidants (e.g., BHT, pyrogallol, ascorbyl palmitate) may also be used, especially when the analysis is prolonged. They can be added during sample disintegration or saponification, or added to solvents (e.g., THF), standard solutions, and isolates.

Exposure to light, especially direct sunlight or UV light, induces trans cis photo-isomerization and photo-destruction of carotenoids. Thus, carotenoid work must be done under subdued light. Open columns and vessels containing carotenoids should be wrapped with aluminum foil, and TLC development tanks should be kept in the dark or covered with dark cloth or aluminum foil. Because of their thermo-lability, carotenoids should be heated only when absolutely necessary.

Carotenoid extracts or solutions should be concentrated in a rotary evaporator at reduced pressure, at a temperature below 40^{o}C, and solvent evaporation should be finished with N_2 or argon. Carotenoids may decompose, dehydrate, or isomerize in the presence of acids. Most carotenoids are stable under alkali conditions. A neutralizing agent (e.g., $CaCO_3$, $MgCO_3$, or $NaHCO_3$) may be added during extraction to neutralize acids liberated from the food sample itself. Finally, reagent-grade, UV/VIS grade or HPLC grade solvents should be employed. Any impurities can affect the carotenoid analysis.

1.5. Research Need in This Area

Many recent papers show the important role of bioactive phytochemicals to maintain a good health status. Among them the carotenoids are the best known. Researchers have postulated that many chronic diseases; cardiovascular disease, cancer, diabetes, eye diseases and aging itself are the result of long-term oxidative stress. It is known that carotenoids have the ability to quench singlet oxygen due to their conjugated double bond system and the maximum efficiency being shown by carotenoids is with nine or more conjugated double bonds.

Apart from several kinds of foods, microalgae have become a potential source of carotenoids. Microalgae are an extremely heterogeneous group of organisms. To be called a microalga, the organism needs to be small (usually microscopic), unicellular (but can be colonial with little or no cell differentiation), colorful (due to photosynthetic

and accessory pigments), occur mostly in water (but not necessarily) and most likely be photoautotrophic (but not necessarily all the time).

This very diversity makes microalgae, as a group, a potentially rich source of a vast array of chemical products with applications in the feed, food, nutritional, cosmetic, and pharmaceutical and even fuel industries. There are very few commercial microalgal high value products in the market today (e.g. fatty acids (FAs), and carotenoids). We hope that future drugs developed from microalgal products might be prepared and packaged as other pharmacological compounds. This is perhaps the most promising aspect of microalgal biotechnology.

The need for reliable data on food carotenoids is widely acknowledged in the international literature in different fields. Trends in carotenoid analysis of foods reflects not only advances in analytical methodology and instrumentation, but also greater understanding of the role of these compounds in human health. For a long time carotenoid analysis involved quantification of only the major provitamin A carotenoids. With the increasing evidence on the importance of carotenoids in reducing the risk of degenerative diseases, carotenoid analysis has gained more attention.

Moreover, one of the great problems of carotenoid analyses lies in the unavailability of standard compounds caused by natural instability of carotenoids. Within this few years, more carotenoid standards are available however their prices are extremely high. For this reason, especially accumulated carotenoids in microalgae can also be an alternative source for standards apart from their roles in diseases as many microalgae have the ability to involve different types of carotenoids.

It can be said that, carotenoids have been intensely studied by organic chemists, food chemists, biologists, physiologists, medical doctors and recently also by environmentalists and great demands have been placed on their identification and determination (Ladislav 2005).

1.6. Aim of this work

The goal of this work is to identify and determine the concentration of carotenoids and that are produced by selected microalgae from EGE-MACC (Ege-Microalgae Culture Collection) by HPLC-DAD and some spectrometric techniques.

First, the microalgae will be cultivated at laboratory conditions in their natural environments and they will be harvested when sufficient biomass obtained. For this purpose, *Prochlorococcus sp.*, *Scenedesmus protuberans* and *Nitzschia sp.* were selected whose carotenoid contents have not been not studied in literature so far.

Second, the extraction procedure will be developed. After, the identification of carotenoids will be done using chromatographic and spectrometric techniques followed by their determination.

Then, the study is planned to be directed towards the highly accumulated carotenoid in order to achieve its highest amount. Therefore, solvent extraction parameters will be optimized.

Finally, some parameters like content of culture medium, light and oxidative stress conditions will be tried since it is known that microalgae produce more/different carotenoids when exposed to different conditions. Therefore, the carotenoid profiles of microalgae cultivated at normal conditions will be compared to these stress conditions in order to get the highest recovery of carotenoids or an individual carotenoid.

CHAPTER 2

MATERIALS AND METHODS

2.1. Chemicals

All-trans-lutein, all-trans-astaxanthin were obtained from Dr. Ehrenstorfer GmbH, trans-β-Apo-8′carotenal, all-trans-fucoxanthin and pyrogallol were purchased from Sigma-Aldrich. In addition, all-trans-violaxanthin, all-trans-neoxanthin, all-trans-beta-carotene, all-trans-alpha-carotene and 9-cis-beta-carotene were provided from Carotenature. All the solvents used in this study were HPLC-grade and obtained from Merck.

2.2. Instrumentation

Agilent 1200 Series HPLC-DAD system was used for the determination of carotenoids. Prior to use, all HPLC-grade mobile phases were degassed using an (Elmasonic S80H) ultrasonic bath. For the LC-MS analyses, AB Sciex Api 4000 Q-trap was used.

The reversed phase column selected for use was a YMC Carotenoid C_{30}, 250 mm x 4.6 mm, 5µm (Waters, Milford, MA, USA). The mobile phase used for the separation of carotenoids from green microalgae consisted of methanol, methyl-tert-butyl ether and water containing 0.10% TEA and 0.010% pyrogallol with a gradient program at a flow rate of 1.0 mL min^{-1}. On the other hand, methanol and acetonitrile containing 0.10% TEA and 0.010% pyrogallol were used to separate the carotenoids in brown microalgae using isocratic elution with a flow rate of 1.0 mL min^{-1}. The injection volume was 20.0 µL and detection was made by diode array detector with quantitation at 450 nm. The column temperature was set at 25.0^{o}C. The spectra from 300 to 600 nm was recorded and stored for tentative identification of other carotenoids. Extraction of carotenoids was performed by using ultrasonic bath. In addition, the solvents were removed by (Heidolph Hei-VAP Advantage) rotary evaporator.

2.3. Microalgal Strains and Their Cultivation

Scenedesmus protuberans, Prochlorococcus sp. and *Nitzschia sp.* were selected from EGE-MACC (Ege-Microalgae Culture Collection). Culture conditions were optimized for these microalgae. Green microalgae have been cultivated in Bold's Basal (BB) Medium. The content of this medium is given in Table 2.1. On the other hand, *Nitzschia sp.* was grown in F/2 medium the contents of which are shown in Table 2.2. The reason why different media has been chosen is that they grow very quickly in the selected medium. The microalgal cells in 2.0 L bottles were illuminated with the continous light of 2000 lux at 24.0^{o}C. Ventilation rates were 1.25 vvm. Cultures were prepared from 10 % inoculation from a stock 4-days 10% inoculation. Illumination was provided by using cool white fluorescent lamps (Philips, 18 W/54). All glassware and the medium were sterilized in autoclave at 121.0^{o}C for 20.0 minutres prior to use.

Table 2.1. Content of Bold's Basal (BB) Medium

Contents	**Volumes**
$NaNO_3$ (5.0g/200mL)	10.0 mL
$CaCl_2.2H_2O$ (0.5g/200mL)	10.0 mL
$MgSO_4.7H_2O$ (1.5g/200mL)	10.0 mL
K_2HPO_4 (Dibasic) (1.5g/200mL)	10.0 mL
KH_2PO_4 (3.5g/200mL)	10.0 mL
NaCl (0.5g/200mL)	10.0 mL
$FeSO_4.7H_2O$ (4.98g/L)	1.0 mL
H_3BO_3 (11.42g/L)	1.0 mL
KOH solution	1.0 mL
Trace Metals	1.0 mL
Distilled water to	1.0 L

Table 2.2. Content of F/2 Medium

F/2 Medium	
Chemicals	**g/1000.0 mL Seawater**
$NaNO_3$	0.075
$Na_2HPO_4.7H_2O$	0.005
$Na_2SiO_3.9H_2O$	0.03
Tiamin-HCl	0.0001
Biotin	0.0005
Vitamin B_{12}	0.00005
Trace Elements	**1.0 mL g/1000.0 mL Distilled water**
Na_2EDTA	4.36
$FeCl_3.6H_2O$	3.15
$MnCl_2.4H_2O$	0.18
$CuSO_4.5H_2O$	0.01
$ZnSO_4.7H_2O$	0.022
$CoCl_2.6H_2O$	0.01
$NaMoO_4.2H_2O$	0.006

2.4. Preparation of Biomass

When microalgal cultures were ready to harvest, they were filtrated through 0.45 μm Whatman cellulose acetate filter paper. The filtered biomass were washed with deionized water to remove the excess salts that might stem from the culture medium. Harvested cells were stored at −24.0°C and lyophilized accordingly with Christ alpha 1–4 Ld instrument. The lyophilized biomass was then reduced to a size using a mortar and then kept under -20°C prior to extraction of carotenoids. Their optical images were obtained by using a trinocular light microscope (Olympus CH40). SEM characterizations were carried out using Philips XL-30S FEG prior to analysis. In addition, the elemental composition of the microalgae were determined with LECO-932 elemental analyzer and according to the results *Prochlorococcus sp.* mainly involves 47.06% C, 6.92% N, 7.05% H, and 0.64% S by mass, *Scenedesmus protuberans* involves 43.12% C, 5.21% N, 6.51% H, and 0.73% S by mass and *Nitzschia sp* involves 23.2 % C, 3.5% N, 3.5% H, and 0.6% S by mass.

2.5. Preparation of Standard Carotenoid Solutions

Due to the light-sensitive nature of carotenoids preparation of standard solutions was performed under yellow light (Philips lamp TLD 36W/16 yellow, light transmisssion at 500-750 nm) at room temperature within the shortest possible time. For the preparation of carotenoid standards 1.0 mg of each standard was dissolved in 10.0 mL chloroform to produce stock solution which were then diluted to obtain appropriate standard solutions. All of the calibration standards were prepared containing a constant concentration of the internal standard and a variable concentration of caotenoid for the construction of calibration curves. All standard and sample solutions were kept in amber colored volumetric flasks and wrapped by aluminium foil.

2.5.1. Determination of LOD and LOQ

Measurement of the magnitude of analytical background response is performed by analyzing an appropriate number of blank samples and calculating the standard deviation of these responses. Ten repeats of blank sample were tested. Using the standard deviation of the blank sample, the detection and quantification limits were set. The mean of the ten repeats, plus three times the standard deviation, is the detection limit, while ten times the standard deviation is the quantification limit.

2.5.2. Preparation of Standard Curves

Different concentrations (0.010-5.0 mgL^{-1}) of carotenoid standards were injected into the HPLC, and the linear regression equation for standard curve was acquired by plotting the quantity of standard compound injected against their ratio of analyte peak area to the internal standard peak area. All-trans-beta-apo-8'-carotenal was used as internal srandard for the determination of carotenoids in green microalgae whereas all-trans-astaxanthin as used as internal standard for te determination of carotenoids in brown microalga.

2.6. Extraction of Carotenoids from Microalgae

Microalgal powder (0.25 g) were added $CaCO_3$ (0.25 g) and extracted with 10.0 mL of different binary solvent combinations containing 0.010% (w/v) pyrogallol. The mixture was placed in an ultrasonic bath. After the ultrasonic extraction the solution was centrifugated at 6000 rpm for ten minutes and the supernatant was collected. The residue was repeatedly extracted with 10.0 mL extraction solvent until it was colorless. The supernatants were combined, evaporated by the rotary evaporator at 35.0 ^{o}C under vacuum. For the determination of carotenoids in brown microalga, the residue was then dissolved in 10.0 mL of dichloromethane. The samples were diluted fifty times with mobile phase and 20.0 µL were injected for HPLC analysis. The summary of all procedures were shown in Figure 2.2. However, for the determination of carotenoids in green microalgae, the extraction procedure was followed by the saponification. All extraction and work-up procedures were conducted under yellow light to prevent photo-isomerization and degradation of carotenoids.

2.7. Saponification of the Extracts

The saponification procedure was applied only for green microalgae since fucoxanthin in brown microalgae undergoes base catalyzed hydrolysis reaction and turns into fucoxanthinol which affect the yield of extraction as designated in Figure 2.1.

For the colored extracts obtained from green microalgae were then pooled in a separatory funnel containing a more polar solvent like diethyl ether. After gentle shaking, the phases were left to settle and the aqueous phase was removed. This operation should be repeated several times until the upper layer is colorless. The residue obtained was dissolved in 10.0 mL of diethyl ether for partition followed by the addition of 10.0 mL of 10.0% methanolic KOH solution containing 0.01% pyrogallol and saponification was carried out under nitrogen atmosphere. Carotenoids were extracted from the methanolic KOH phase with 10.0 mL diethyl ether and 10.0 mL 10.0% Na_2SO_4 solution. Addition of the salt stops the reaction and also helps the removal of excess alkali. The combined diethyl ether phase was filtered through 0.20 µm nylon membrane filter (Sartorius) and concentrated by rotary evaporator at 25.0^{o}C under 400 mbar.

The remaining water was removed with the aid of nitrogen gas. The residue was then dissolved in 10.0 mL of dichloromethane. The samples were diluted ten times with mobile phase and 20.0 µL were injected for HPLC analysis.

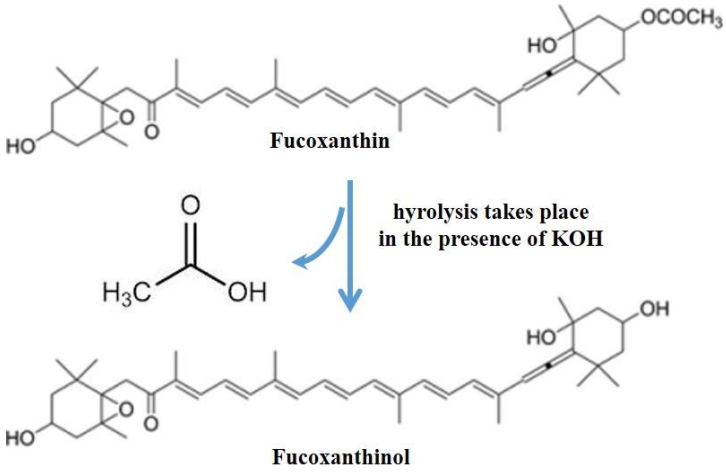


Figure 2.1. Decomposition of fucoxanthin into fucoxanthinol in the presence of basic medium

The summary of all procedures were shown in Figure 2.2. It has been reported that the saponification of the extract of carotenoids has been important procedure for the determination of carotenoids (Britton 1995a). Actually each microorganism has been thought to have differing structures, it should be kept in mind that lutein may have not been in free forms. As Meléndez-Martínez (2007) has pointed out that diol carotenoids like lutein or zeaxanthin may be free or esterified by one or two fatty acids. In addition, saponification reaction eliminates the neutral fats, fatty acids, estres and chlorophylls that might be present in the samples analyzed.

As a consequence, saponification eliminates the unwanted lipids and chlorophylls and hydrolzes carotenoid esters, simplifying the chromatographic separation, identification and quantification of carotenoids in most of the samples. Actually, two microalgae used in this study have distinct structures therefore the saponification procedure should also be optimized for each of them.

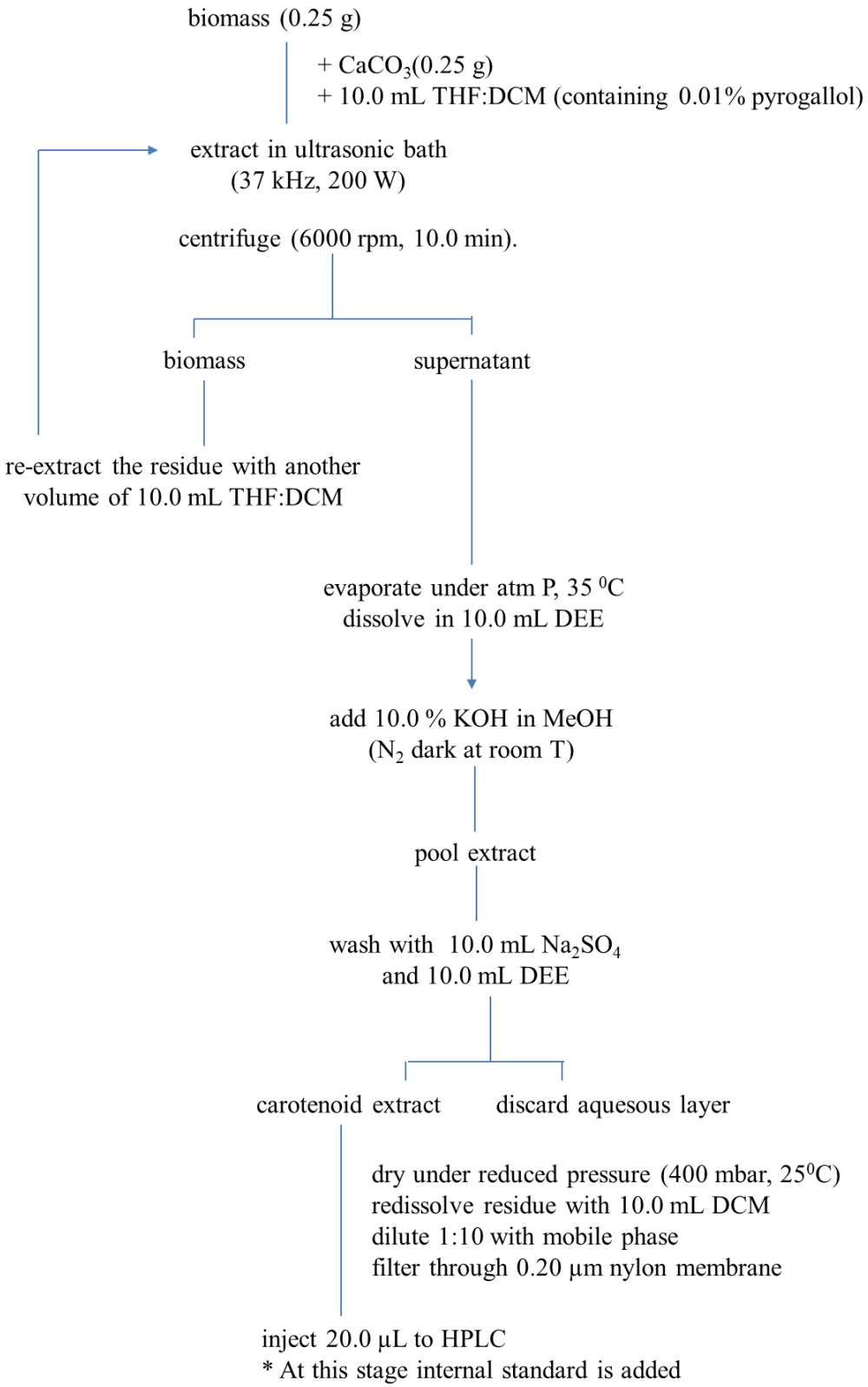


Figure 2.2. Summary of the extraction and saponification method for the analysis of carotenoids in green microalgae *Prochlorococcus sp. and Scenedesmus protuberans*

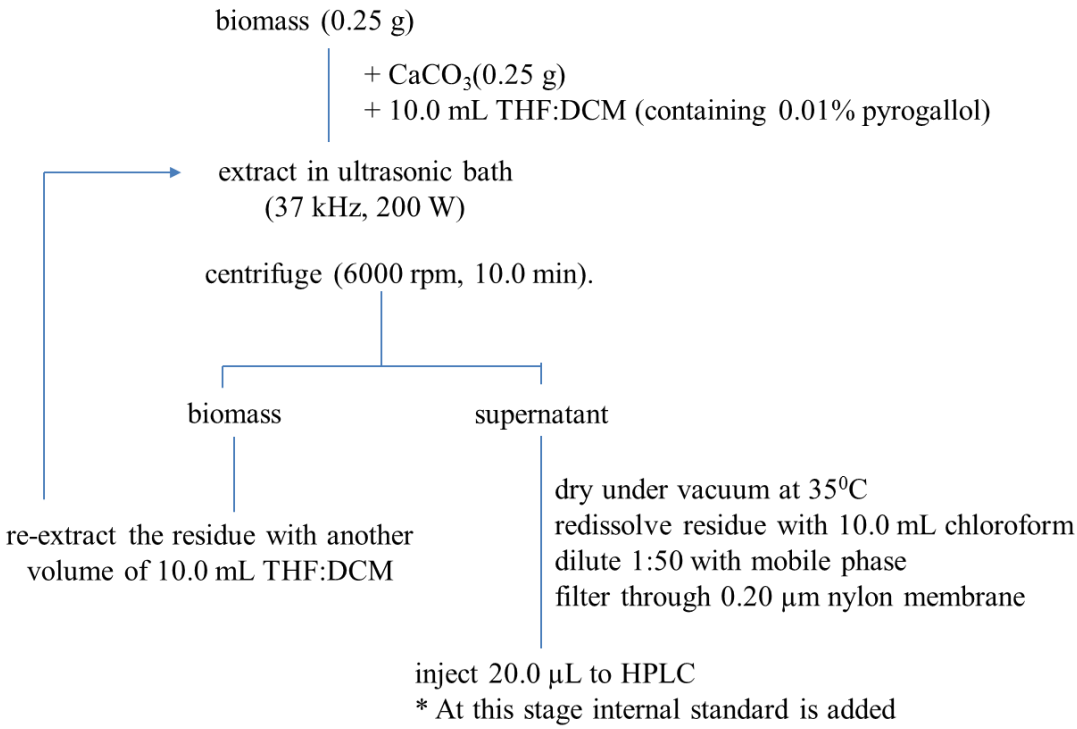


Figure 2.3. Summary of the extraction method for the analysis of carotenoids in brown microalga *Nitzschia sp.*

2.8. Method Development for the Separation of Carotenoids in Microalgae

For the separation of carotenoids in green microalgae, a gradient system was developed due to its resolving power and improved sensitivity as the isocratic elution took much more time. Table 2.3. shows the LC-DAD method developed for the determination carotenoids. On the other hand, isocratic elution using 70% methanol and 30% acetonitrile was applied for the determination of carotenois in brown microalga.

The system suitability was performed using three replicate injections of the standard solutions. Internal standard calibration method was applied. LOD and LOQ values, linearity range and regression equation are all shown in Table 2.3, Table 2.4. and Table 2.5. The developed LC-DAD methods described are fast, specific and precise for the determination of carotenoids in microalgae.

Table 2.3. Gradient program optimized for the analysis of carotenoids by HPLC-DAD

Solvents containing 0.1% TEA			
Time (min)	**MeOH**	**MTBE**	**H_2O**
0	70	25	5
5	60	35	5
10	45	55	0
15	25	75	0

*(Detection was done at 446 nm on C_{30} column with a flow rate of 1.0 mL min^{-1})

2.9. Importance of Lutein

Lutein and its role in the prevention of chronic diseases and health promoting mechanisms has been well documented. It is recommended to prevent some types of cancer (Astorg 1997, Demmig-Adams 2002, Heber and Lu 2002), cardiovascular diseases (Dwyer , 2001), and retinal degeneration (Chiu and Taylor 2007 and Granado 2003). The human body is unable to produce lutein, thus this compound can only be supplied through our diet. As lutein is yellow, it efficiently absorbs the blue light port of the spectrum. Blue light can damage the retina by inducing photoxidative decay. Lutein which is a type of carotenoid acts as blue light filter in the eye. When sufficient levels of lutein are present in the macula, blue light is absorbed and photoxidation is minimized. (Bendich and Oslon 1989, Krinsky , 2003). It is also referred to as the macular pigment. It has been reported that, intake of lutein has been strongly correlated with a decreased risks of cataracts and age-related retinal degeneration. Lutein is a very effective antioxidant that also prevents and reduces the damage caused by free radicals. (Richmond 1990, Le Marchand 1993 and Ziegler 1996).

Naturally occurring lutein is produced mainly in higher plants and algae. Compared with higher plants, algae have an advantage due to its availability for their cultivation in bioreactors on a large scale and thus they can be regarded as a continuous and reliable source of the product (Shi 1997, Shi 1999, Shi 2000). The current commercial source of pure lutein is marigold (*Tagetes erecta L.*). However, the lutein content of marigold flowers is reported to be low which makes alternative lutein rich sources interesting. The use of microalgae in biotechnology has gained a significant attention since they produce great variety of metabolites that are essential to human

health. Among those metabolites, there exist proteins, vitamins, minerals, enzymes, fatty acids, xanthophylls and carotenes during their normal growth phase and when exposed to different enviromental factors (Jin , 2003).

Microalgae exhibit a great metabolic plasticity due to changes in response to environmental conditions sometimes so-called stress factors. Among these, the most important factors that affect lutein content are illumination, temperature, nitrogen availability and source, salinity, the presence of oxidizing substances and the growth rate. It should be emphasized that any of these factors can affect either lutein content or biomass productivity in opposite ways (Fernandez-Sevilla, , 2010). Therefore, it is important that the microalgae should also have a high growth rate even if exposed to stress conditions in order to induce the accumulation of lutein. The interest of lutein from microalgae is still new to the world and this is the main reason for the research on the extraction of lutein is proposed here in this study. Several microalgae have been proposed as potentially useful to produce lutein, such as *Muriellopsis sp.* (Del Campo , 2001, Blanco 2007), *Chlorella zofingensis* (Del Campo 2000), *Chlorella protothecoides* (Wei 2008) or *Scenedesmus almeriensis* (Sáncez 2008). Most of these studies have shown how the culture conditions and biomass productivity were optimized to increase the amount of lutein. Apart from this, it should be noted that solvent extraction conditions may also be a consideration as each microalgal species have different morphologies. There is no exact procedure for the determination of carotenoids or a specific type of carotenoid in microalgae. For this reason, new studies must be directed towards its accurate identification and quantification.

The aims of our further studies are to investigate the optimum solvent extraction conditions for the green microalgae (*Prochlorococcus sp.* and *Scenedesmus protuberans*) in order to achieve the highest amount of lutein and increase its recovery by using different light sources altering the source of nitrogen used and applying oxidative stress in their culture media.

For the extraction method, sonication was preferred as the ultrasound power would agitate the extraction solvent, thus increasing the contact between solvent and targeted compounds, which could greatly improve the extraction efficiency. The accuracy with which individual carotenoids can be identified requires demands on the development of appropriate extraction procedure. The analysis of lutein has been routinely performed by reversed-phase HPLC using C_{30} column because of its improved separation efficiency.

2.9.1. Effect of Extraction Conditions on Lutein Extraction

In order to obtain the highest amount of lutein, it is important to optimize all the possible extraction conditions. For this reason, the effcet of solvent combination, effect of extraction time and saponification time, the effect of temperature and finally the effect of number of extractions were investigated. Moreover, some other conditions were applied to stimulate the biosynthesis of lutein like different nitrogen sources, different types of light and finally different oxidative stress conditions.

2.9.2. Use of Different Nitrogen Sources for Lutein Extraction

In this study, different nitrogen sources were provided such as nitrite, ammonium and urea as previous literature studies have implied that nitrogen content increases the extracted amount of carotenoids like lutein (Shi , 2000). Fernández-Sevilla (2010) has pointed out that nitrogen availability and source are one of the most important factors that affect the lutein content in microalgae. Apart from nitrate nitrite, ammonium and urea have been selected as the different nitrogen sources in order to increase the lutein productivity of novel microalga used in this study. For this reason, equivalent nitrogen concentration has been used in the culture media of the microalga for the comparison of nitrogen sources with the nitrate.

2.9.3. Use of Different Light Sources for Lutein Extraction

In order to investigate the role of light on lutein biosynthesis, cells were grown in batch glass vessels under constant aeration at controlled temperatures. Continuous light was emitted from fluorescent lamps in different colors (white, red and blue) each with 6 W powers.

2.9.4. Use of Oxidative Stress Conditions for Lutein Extraction

The present study also examined whether the use of oxidative stress growth conditions could increase the amount of lutein in green microalgae in BBM medium containing H_2O_2 and NaOCl in the presence of iron as a model for environmental stress. The effect of increasing the concentrations of H_2O_2 and NaOCl over the control was investigated. The $^{\bullet}OH$ and $^1O_2$ are the two most powerful oxidative species in nature and generated based on the Eqs (1-4) (Ip , 2005 and Shi ,1999) given below:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + \bullet OH \quad (1)$$

$$NaClO + H_2O \rightarrow HOCl + Na^+ + OH^- \quad (2)$$

$$HOCl + Fe^{2+} \rightarrow Fe^{3+} + Cl^- + \bullet OH \quad (3)$$

$$H_2O_2 + NaClO \rightarrow H_2O + NaCl + {}^1O_2 \quad (4)$$

2.10. Importance of Fucoxanthin

Fucoxanthin, a yellowish brown carotenoid, is produced specifically in edible brown algae and attracts much attention because of its beneficial effects on human health. It has been described that fucoxanthin has a number of therapeutic activities including anticancer, antihypertensive, anti-inflammatory, and antiobesity effects (Peng 2011). Its significant biological properties are reported to be due to its unique molecular structure (Nomura , 1997). It has an unusual allenic bond and some oxygenic functional groups such as epoxy, hydroxyl, carbonyl and carboxyl parts in its structure. This allenic bond is addressed to be responsible for its higher antioxidant behaviour (Sachindra 2007).

Extraction of fucoxanthin from microalgae is a new interest in the related community. Upto now, most of the studies including the industrial production are conducted with the extraction of fucoxanthin from brown seaweeds (Fung 2013, Maeda 2005, Jaswir 2011, Zailanie 2011, Yan 1999). Despite their roles and great abundance, information on the biosynthesis of carotenoids (or a specific carotenoid) is still incomplete. Therefore, new studies must be directed towards their identification and determination (Peng 2011, Mise 2011, Jaswir 2011). Solvent extraction conditions may also be a consideration as each microalgal species have different morphologies.

In the following studies, extraction/production of fucoxanthin from a novel brown microalga, *Nitzschia sp.* was investigated to improve the extraction efficiency under various conditions. We aimed to find out the optimum extraction conditions and increase its recovery by altering the nitrogen source used, using different light sources or introducing oxidative stress into the culture medium.

2.10.1. Effect of Extraction Conditions on Fucoxanthin Extraction

For the extraction of fucoxanthin from brown microalga, the effect of solvent combination, effect of extraction time, the effect of temperature and finally the effect of number of extractions were examined. As in the case of lutein, some other conditions were applied to stimulate the biosynthesis of fucoxanthin like different nitrogen sources, different types of light and finally different oxidative stress conditions.

2.10.2. Use of Different Nitrogen Sources for Fucoxanthin Extraction

In literature, it has not been reported that different nitrogen sources have effect on fucoxanthin amount. Therefore, nitrite, ammonium and urea have been selected as the different nitrogen sources in order to increase the fucoxanthin productivity of novel microalga used in this study. For this reason, equivalent nitrogen concentration has been used in the culture media of the microalga for the comparison of nitrogen sources with the nitrate.

2.10.3. Use of Oxidative Stress for Fucoxanthin Extraction

The oxidizing capacity of some species is so strong that they are considered highly reactive oxygen species (ROS). Hydrogen peroxide is a product of microalgae via its oxidative metabolism. Nearly all living things decompose low concentrations of hydrogen peroxide to water and oxygen. However, H_2O_2 can injure cells at high concentrations or lead to acclimation at moderate levels. Particularly in the presence of iron, hydrogen peroxide decomposes and generates the highly reactive hydroxyl radical through Fenton reaction. As with many other organisms microalgae develop defence

mechanisms against high levels of ROS and these factors can affect either content or biomass productivity in opposite ways (Fernandez-Sevilla ,2010).

2.10.4. Use of Different Light Sources for Fucoxanthin Extraction

For the investigation of different light sources (white, red and blue) brown microalga was illuminated with continous fluorescent lamps inserted in the bottles each with 6 W powers. In each of the case the growth rate of microalga and fucoxanthin accumulated were found.

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Growth rates and Morphologies of Microalgae

When the appropriate medium was used, the growth rates were found to be 0.110 day^{-1}, 0.419 day^{-1} and 0.134 day^{-1} for *Scenedesmus protuberans*, *Prochlorococcus sp.* and *Nitzschia sp.*, respectively. Growth rates for each microalga were calculated according to Becker's formula (Equation 1) using the data obtained by the absorbance values taken at 450 nm and the growth curves for the green microalgal growth were shown in Figure 3.1 and brown microalgal growth were designated in Figure 3.2.

$$\mu = \frac{\ln x_2 - \ln x_1}{\Delta t} \quad (1)$$

μ : specific growth rate

x_2 : cell concentration at time t_2

x_1 : cell concentration at time t_1

Δt : t_2-t_1

As it can be derived from the curves that, each microalga has different growth characteristics. *Scenedesmus protuberans* has the highest growth rate among all the microalgae. Growth rate is very important factor in terms of biomass productivity. If a biomolecule is going to be isolated from a microalga, both the growth rate of microalgae and accumulation of the desired biomolecule are significant.

Another important factor is that the morphology of a microalga. As it can be designated in Figure 3.3. and Figure 3.4. each microalgae has different morphologies which means that they also have different cell walls. This, in turn affects the efficient extraction of biomolecules from the species. In order to obtain the targeted molecules after the extraction, the cell wall thus the microalga should be destroyed and dissolved in the appropriate solvent. for its destructive power, ultrasound assisted extarction was used for the extraction of carotenoids from all the microalgae.

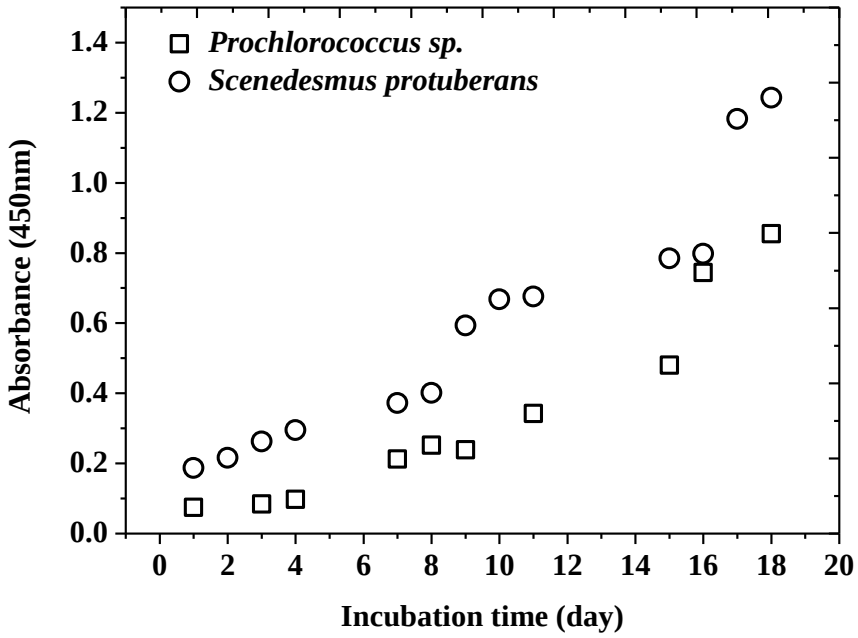


Figure 3.1. Growth curves for Green Microalgae

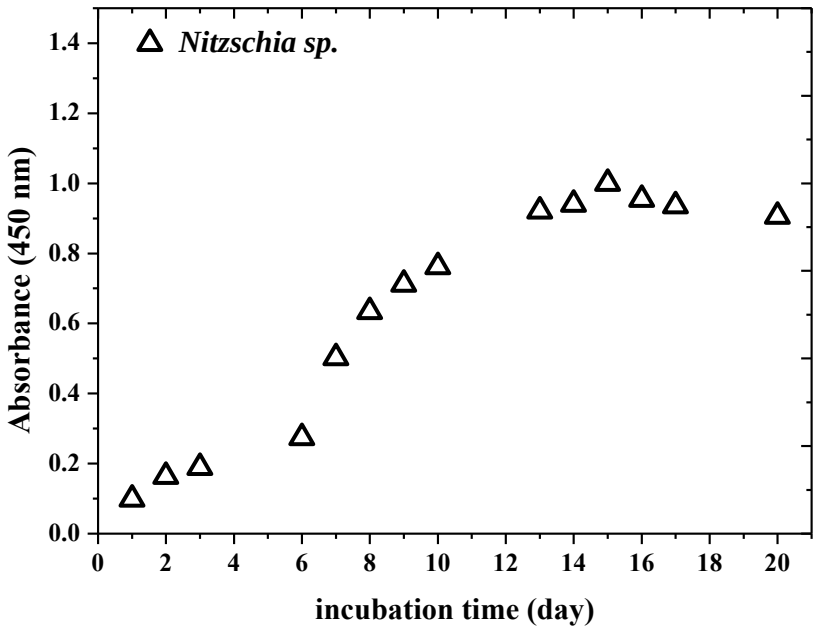


Figure 3.2. Growth curves for Brown Microalga

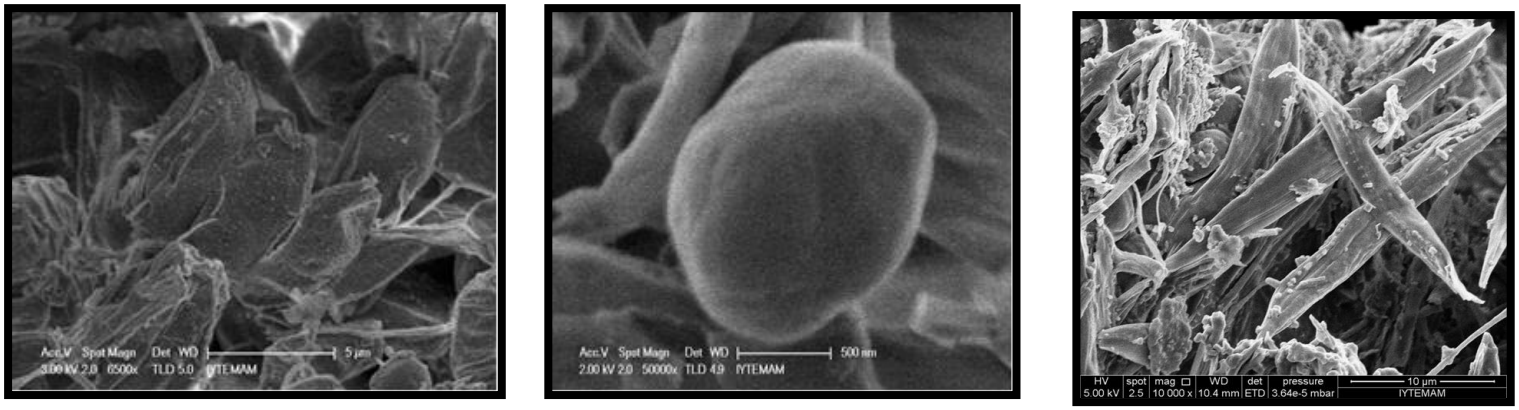


Figure 3.3. SEM images for (a) *Scenedesmus protuberans* (×6500) and (b) *Prochlorococcus sp* .(×50000), (c) *Nitzschia sp.* (x10000)

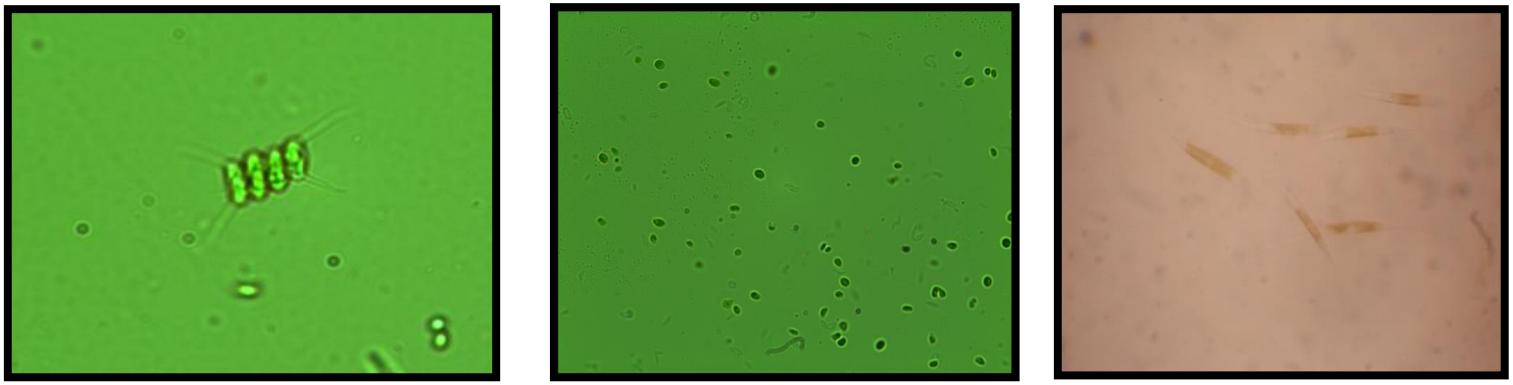


Figure 3.4. Optical images for (a) *Scenedesmus protuberans* (x40), (b) *Prochlorococcus sp.* (x40), and *Nitzschia sp.* (x40)

3.2. HPLC-DAD Analysis of Carotenoids in Green Microalgae

The multidimensional nature of diode array spectra makes it an extremely significant tool for the study of complex products such as carotenoids. The simultaneous acquisition of complete spectra as peaks elute also provides sufficient information for tentative peak identification by comparing the spectrum of the unknown to that of known compounds. Acording to the HPLC chromatograms obtained, some of the peaks were tentatively identified on the basis of spectral matching observed versus literature data (Britton 2004). According to the chromatogram obtained from *Scenedesmus protuberans*, (Figure 3.6) there appears a similarity between the carotenoid profile of *Prochlorococcus sp*. However, chlorophyll b was observed in the latter due to shorter saponification time. Yet, the presence of chlorophyll b does not seem to make the chromatogram complicated.

Light-absorption properties provide one of the first criteria for identification and characterization of carotenoids. Because of their long conjigated double-bond system (the chromophore), the carotenoids absorb light strongly and exhibit intense main absorption bands in UV-VIS region. Actually, three features of the UV-VIS spectrum provide valuable information for structural characterization and quantitative analysis; (1) wavelength of maximum absorption, (2) spectral shape and (3) intensity of absorption (Britton 2004). This data then combined with the values obtained by mass spectrometric data. Figure 3.11, Figure 3.12 and Figure 3.13 show the absorbance spectra for carotenoid standards and carotenoids extracted from microalgae, *Prochlorococcus sp., Scenedesmus protuberans* and *Nitzschia sp.* respectively. In addition, Table 3.1, Table 3.2 and Table 3.3 show the characteristic absorption wavelengths of carotenoids identified for each microalga which gives important clues about the identification. According to the data, carotenoid standard values well match with the observed values.

The employed chromatographic conditions can also separate many of the carotenoid compounds within 15.0 min. Xanthophylls were eluted in the first 10 minutes and then carotenes were observed in the chromatograms of microalgae (Figure 3.5 and Figure 3.6). In these chromatograms, carotenoids were identified by comparison of their spectroscopic data obtained by diode array detector throughout the analysis and then these values were

compared with the literature values reported (Britton 2004). Even small differences in their absorption profiles give us an idea about the identity of each carotenoid.

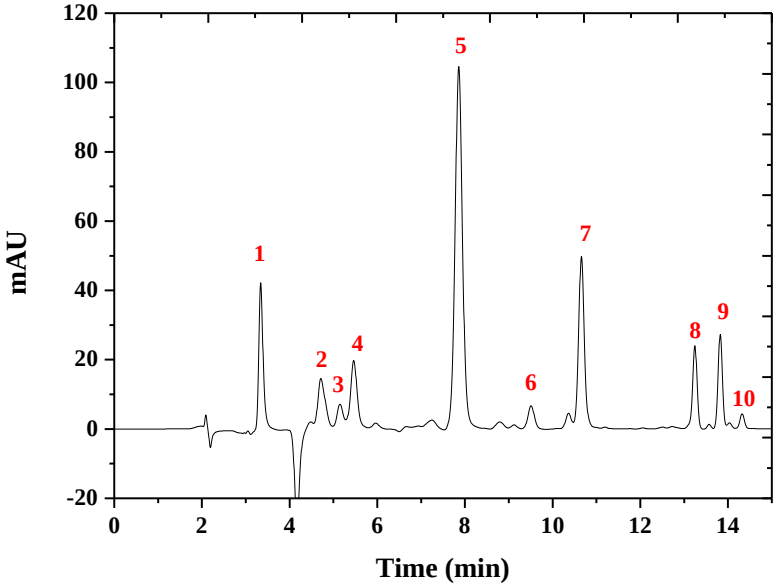


Figure 3.5. HPLC chromatogram for *Prochlorococcus sp.* obtained at 450 nm (1) chlorophyll b, (2) neoxanthin, (3) violaxanthin, (4) 9- or 9-cis-violaxanthin (5) lutein, (6) 9- or 9-cis-lutein, (7) trans-beta-apo-8'-carotenal, (8) α -carotene, (9) β-carotene, (10) 9- or 9-cis-carotene.

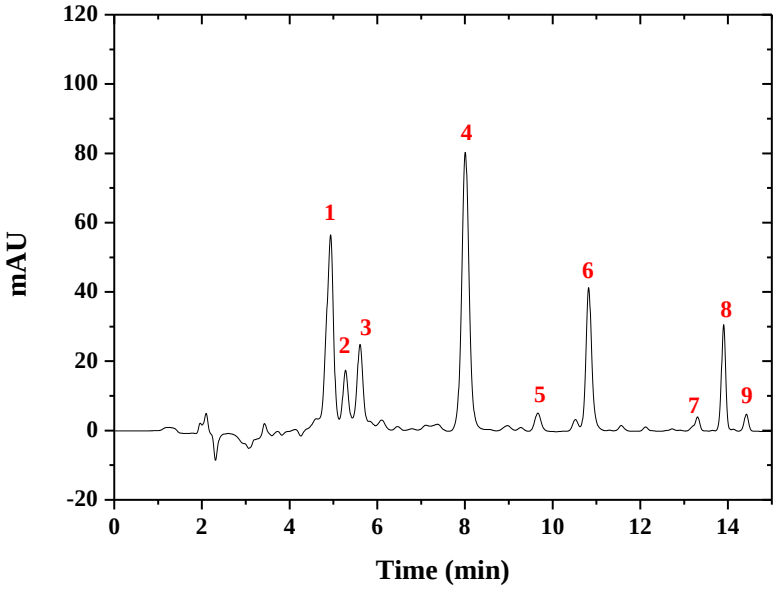


Figure 3.6. HPLC chromatogram for *Scenedesmus protuberans* obtained at 450 nm (1) loroxanthin, (2) violaxanthin, (3) 9- or 9-cis-violaxanthin (4) lutein, (5) 9- or 9-cis-lutein, (6) trans-beta-apo-8'-carotenal, (7) α -carotene, (8) β –carotene, (9) 9- or 9-cis-carotene.

3.3. HPLC-DAD Analysis of Carotenoids in Brown Microalga

A simple the isocratic elution was developed for the determination of fucoxanthin. The multidimensional nature of diode array spectra makes it an extremely significant tool for the study of complex products such as carotenoids. The simultaneous acquisition of complete spectra as peaks elute also provides sufficient information for tentative peak identification by comparing the spectrum of the unknown to that of known compounds. Figure 3.7 demonstrates the HPLC chromatogram for HPLC-DAD analysis of carotenoids in brown microalga It has been certainly seen that they are well separated form each other. In this chromatogram, peaks eluting in the few minutes are due to chlorophyl b. It could be easily understood by comparison of its spectroscopic behaviour (data not shown) obtained by diode array detector.

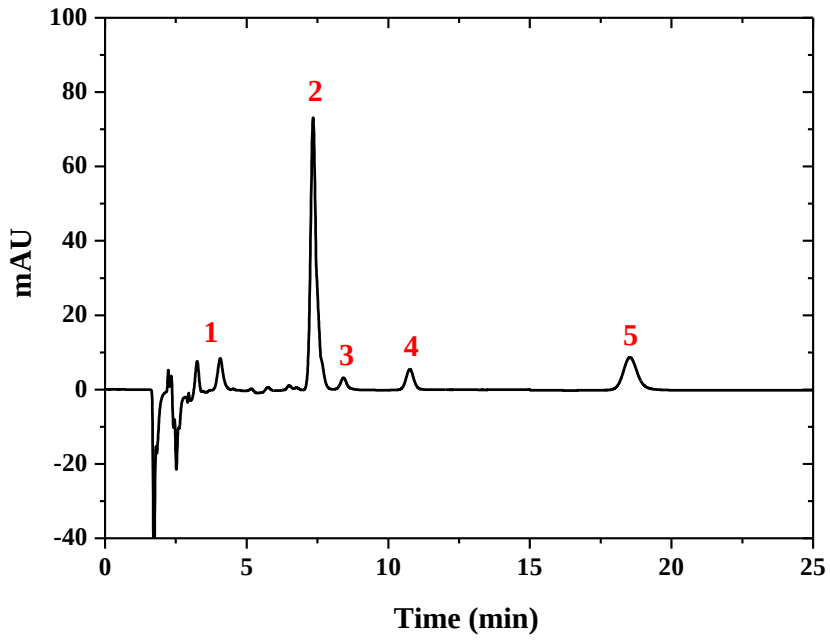


Figure 3.7. HPLC chromatogram for *Nitzschia sp*. obtained at 450 nm (1) chlorophyll b (2) fucoxanthin, (3) fucoxanthinol (4) neoxanthin, (5) astaxanthin

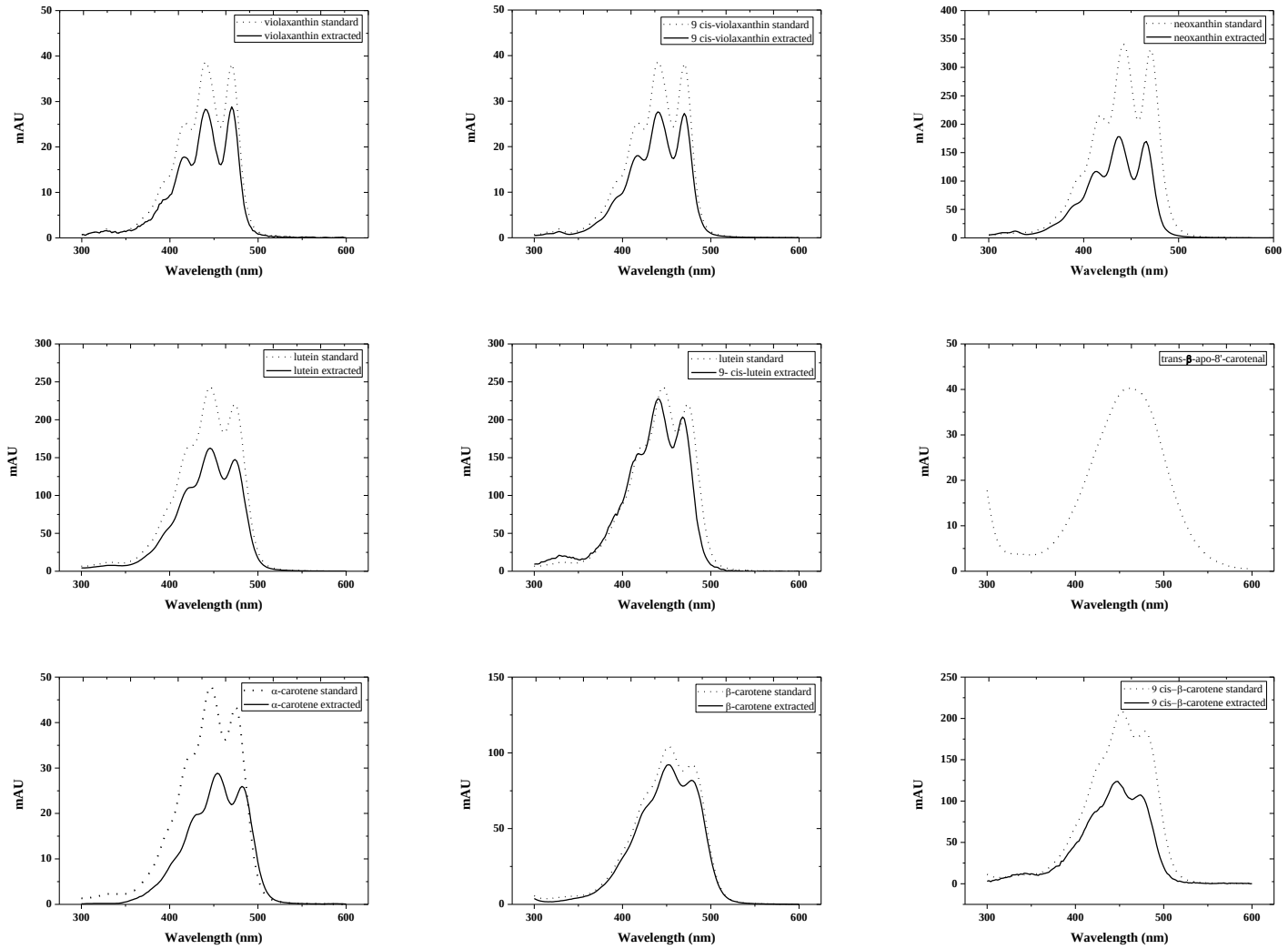


Figure 3.8. Absorbance spectra for available carotenoid standards and carotenoids extracted from *Prochlorococcus sp.*

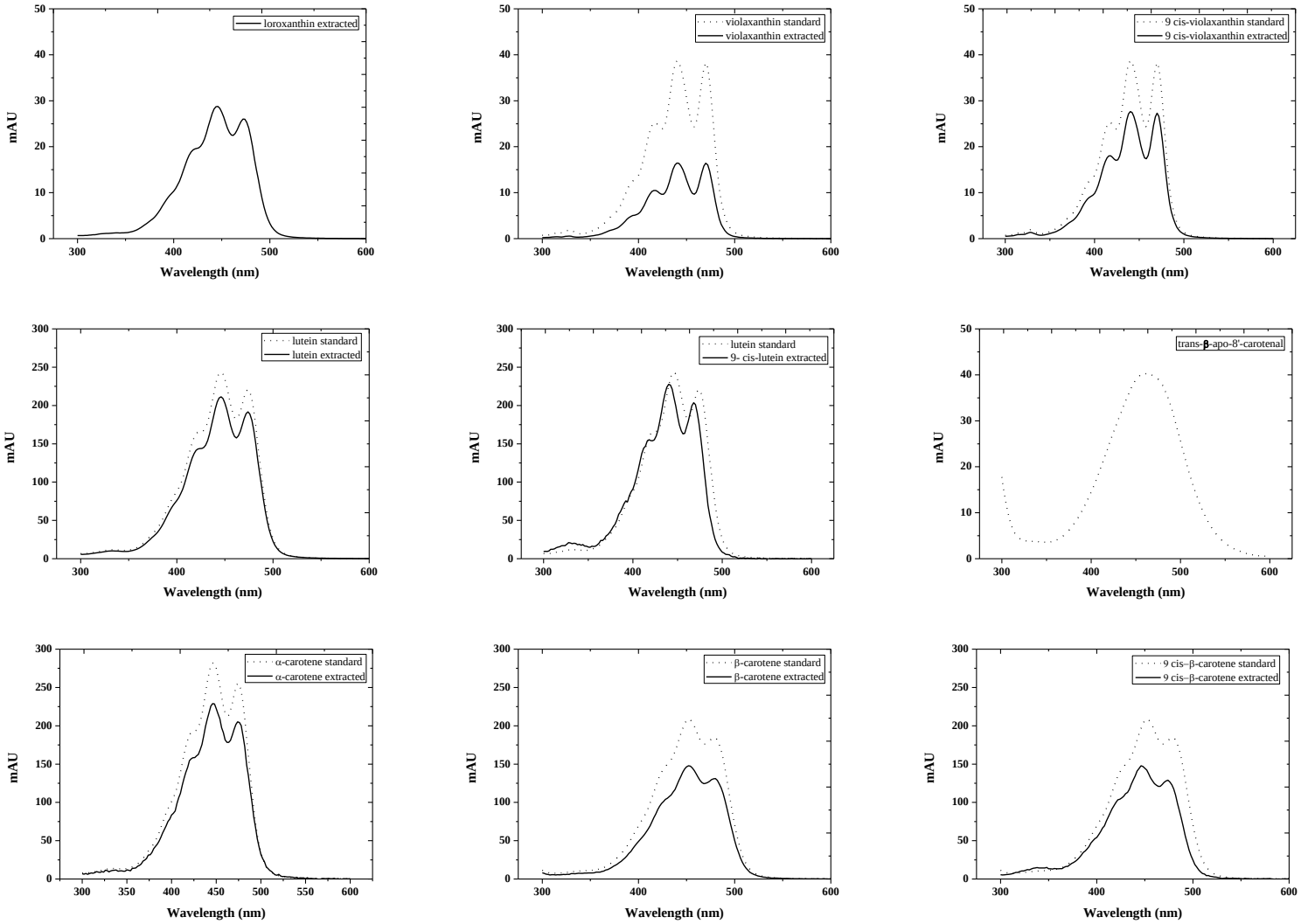


Figure 3.9. Asorbance spectra for available carotenoid standards and carotenoids extracted from *Scenedesmus protuberans*

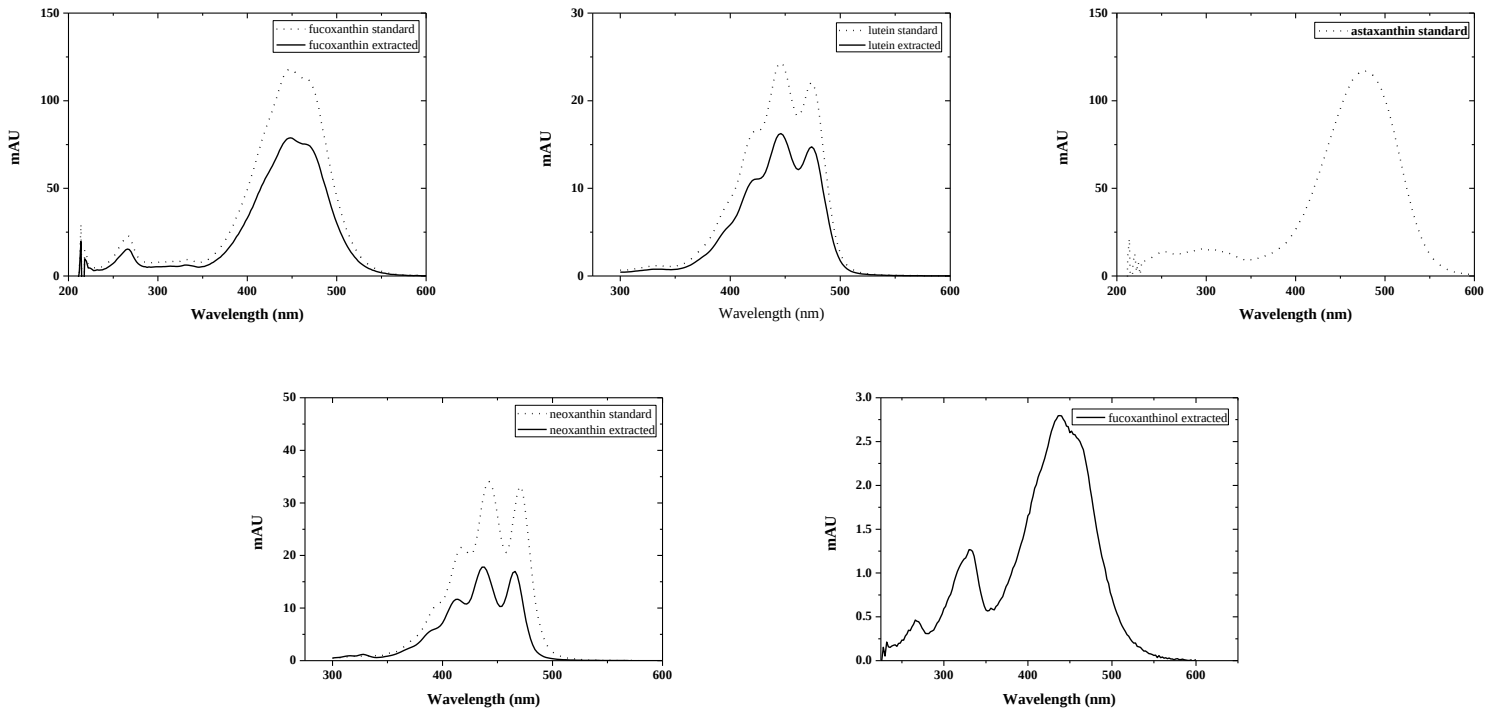


Figure 3.10. Asorbance spectra for available carotenoid standards and carotenoids extracted from *Nitzschia sp.*

Table 3.1. Tentative identification data for carotenoids in *Prochlorococcus sp.*

Peak no	Compound	λ(reported)*			λ(observed)			λ(standards)		
1	Chlorophyll b		452			453				
2	Neoxanthin	418	442	471	418	440	471	418	440	471
3	Violaxanthin	419	440	470	417	440	470	418	440	470
4	9-or 9'-cis-violaxanthin	414	436	464	418	440	470			
5	Lutein	422	445	474	423	446	474	424	446	474
6	9-or 9'-cis-lutein	420	442	470	418	440	470			
7	**Internal standard		458			456			456	
8	α -carotene	423	444	473	423	446	474	423	446	474
9	β -carotene	428	450	478		452	478		452	478
10	9- or 9-cis-carotene	422	448	474	423	446	474	423	446	474

* Britton 2004
** trans-beta-apo-8'-carotenal

Table 3.2. Tentative identification data for carotenoids in *Scenedesmus protuberans*

Peak no	Compound	λ(reported)*			λ(observed)			λ(standards)		
1	Loroxanthin		446	474		446	474			
2	Violaxanthin	419	440	470	417	440	470	418	440	470
3	9-or 9'-cis-violaxanthin	414	436	464	418	440	470			
4	Lutein	422	445	474	423	446	474	424	446	474
5	9-or 9'-cis-lutein	420	442	470	418	440	470			
6	**Internal standard		458			456			456	
7	α -carotene	423	444	473	423	446	474	423	446	474
8	β -carotene	428	450	478		452	478		452	478
9	9- or 9-cis-carotene	422	448	474	423	446	474	423	446	474

* Britton 2004
** trans-beta-apo-8'-carotenal

Table 3.3. Tentative identification data for carotenoids in *Nitzschia sp.*

Peak no	Compound	λ(reported)			λ(observed)			λ(standards)		
1	Chlorophyll b		452			453				
2	Fucoxanthin	420	444	467	418	446	467	418	446	467
3	Fucoxanthinol		452		438	452			438	452
4	***Internal standard		478			478			478	
5	Neoxanthin	418	442	471	418	440	471	418	440	471

* Britton 2004
*** astaxanthin

3.4. LC/MS (APCI) Analysis of Carotenoids in Microalgae

In HPLC, UV–VIS instruments are the most common detectors used to identify carotenoids. However, given that the UV–VIS spectra of many carotenoids are similar and a number of structurally related molecules co-elute, many researchers have complemented the identification of carotenoids using other detection methods (Su , 2002). Among those, mass detectors have shown great advantages for the analysis of these substances, including the elucidation of their structure on the basis of the molecular mass and their fragmentation pattern. Several ionization methods have been reported for MS analysis of carotenoids, including electron impact (EI), fast atom bombardment (FAB), matrix-assisted laser desorption/ionization (MALDI), electrospray (ESI), atmospheric pressure chemical ionization (APCI) and more recently, atmospheric pressure photoionization (APPI) and atmospheric pressure solids analysis probe (ASAP). Most mass spectra of carotenoids have been acquired using positive ion mode. However, APCI has become the most widely used ionization technique for carotenoids and shows high sensitivity for their analysis (Rivera 2011, Hao 2005). APCI has been used to successfully ionize not only xanthophylls and carotenes but also carotenoid esters (Rezanka 2009, Kurz 2008, Weller 2003).

Although the fragment pattern observed in the carotenoid mass spectra depends on the ionization technique and composition of the mobile phase used, characteristic carotenoid fragments have been observed with various ionization techniques. For example, the ions $[M-92]^{+\bullet}$, $[M-92]^{+}$ and $[M+H-92]^{+}$ correspond to loss of a neutral molecule of toluene and indicate the presence of extensive conjugation within the molecule. These ions have been obtained by using EI, ESI (Rivera 2011) and APCI (Breeman 1996, Rezanka , 2009 and Rosso , 2007).

The ions $[M-106]^{+\bullet}$, $[M-106]^{+}$ and $[M+H-106]^{+}$ are also very characteristic in carotenoids and it is explained by the removal of the xylene molecule from the polyene chain. In addition, the ions $[M-69]^{+\bullet}$, $[M-69]^{-}$ and $[M-69]^{+}$ have been described using EI and APCI (Rosso , 2007). The removal of a hydroxyl group or a molecule of water $[M-17]^{+\bullet}$ or $[M+H-18]^{+}$ is characteristic of the presence of a hydroxyl group in the compound. These ions have been obtained through EI, ESI and APCI (Breeman 1996).

Table 3.4. LC-MS-APCI data of carotenoids in *Prochlorococcus sp.*

Peak No	Carotenoid	m/z found	m/z reported
2	Neoxanthin	601.5 $[M+H]^+$, 583.5 $[M+H-H_2O]^+$, 565.4 $[M+H-2H_2O]^+$	601 $[M+H]^+$, 583 $[M+H-H_2O]^+$, 565 $[M+H-2H_2O]^+$
3	Violaxanthin	601.5 $[M+H]^+$, 583.5 $[M+H-H_2O]^+$,509.4 $[M+H-92]^+$	601 $[M+H]^+$, 583 $[M+H-H_2O]^+$,509 $[M+H-92]^+$
4	9-or-9'cis-violaxanthin	601.5 $[M+H]^+$	601 $[M+H]^+$
5	Lutein	569.5 $[M+H]^+$, 551.5 $[M+H-H_2O]^+$	569 $[M+H]^+$, 551 $[M+H-H_2O]^+$
6	9-or 9'-cis-lutein	569.5 $[M+H]^+$	569 $[M+H]^+$
8	α-carotene	537.4 $[M+H]^+$, 519.4 $[M+H-H_2O]^+$	537 $[M+H]^+$, 519 $[M+H-H_2O]^+$
9	β-carotene	537.4 $[M+H]^+$, 519.4 $[M+H-H_2O]^+$	538 $[M+H]^+$, 519 $[M+H-H_2O]^+$
10	9-or 9'-cis-β-carotene	537.4 $[M+H]^+$, 519.4 $[M+H-H_2O]^+$	539 $[M+H]^+$, 519 $[M+H-H_2O]^+$

Table 3.5. LC-MS-APCI data of carotenoids in *Scenedesmus protuberans*

Peak No	Carotenoid	m/z found	m/z reported
1	Loroxanthin	585.5 $[M+H]^+$, 567.4 $[M+H-H_2O]^+$, 549.5 $[M+H-2H_2O]^+$	585 $[M+H]^+$, 567 $[M+H-H_2O]^+$, 549 $[M+H-2H_2O]^+$
2	Violaxanthin	601.5 $[M+H]^+$, 583.5 $[M+H-H_2O]^+$, 509.4 $[M+H-92]^+$	601 $[M+H]^+$, 583 $[M+H-H_2O]^+$, 509 $[M+H-92]^+$
3	9-or-9'cis-violaxanthin	601.5 $[M+H]^+$	601$[M+H]^+$
4	Lutein	569.5 $[M+H]^+$, 551.5 $[M+H-H_2O]^+$	569 $[M+H]^+$, 551 $[M+H-H_2O]^+$
5	9-or 9'-cis-lutein	569.5 $[M+H]^+$	569 $[M+H]^+$
7	α-carotene	537.4 $[M+H]^+$, 519.4 $[M+H-H_2O]^+$	537 $[M+H]^+$, 519 $[M+H-H_2O]^+$
8	β-carotene	537.4 $[M+H]^+$, 519.4 $[M+H-H_2O]^+$	538 $[M+H]^+$, 519 $[M+H-H_2O]^+$
9	9-or 9'-cis-β-carotene	537.4 $[M+H]^+$, 519.4 $[M+H-H_2O]^+$	539 $[M+H]^+$, 519 $[M+H-H_2O]^+$

Table 3.6. LC-MS-APCI data of carotenoids in *Nitzschia sp.*

Peak No	Carotenoid	m/z found	m/z reported
2	Fucoxanthin	658.7 $[M+H]^+$, 640.7 $[M+H-H_2O]^+$, 622.8 $[M+H- 2H_2O]^+$	659 $[M+H]^+$, 641 $[M+H-H_2O]^+$, 623 $[M+H-2H_2O]^+$
3	Fucoxanthinol	617.6 $[M+H]^+$, 599.6 $[M+H-H_2O]^+$, 581.8 $[M+H- 2H_2O]^+$	617 $[M+H]^+$, 599 $[M+H\ H_2O]^+$, 581 $[M+H-2H_2O]^+$
4	Neoxanthin	601.6 $[M+H]^+$, 583.5 $[M+H-H_2O]^+$, 565.5 $[M+H-2H_2O]^+$	601 $[M+H]^+$, 583 $[M+H-H_2O]^+$, 565 $[M+H-2H_2O]^+$

Thus, although some carotenoids show the same or a very similar fragmentation pattern (meaning that their structures are similar and therefore they might co-elute), differences between the intensities of their fragments have been reported.

In order to help the identification of carotenoids combined with the spectral data obtained from DAD detector, possible carotenoids are defined. The values of molecular ions are looked for each possible carotenoid in the mass spectrum of the extracts as they are shown in Figure 3.11, Figure 3.2 and Figure 3.13. The LC-MS-APCI data of carotenoids for the microalgae (*Prochlorococcus sp, Scenedesmus protuberans* and *Nitzschia sp.*, respectively) were compared with the data given in literature as shown in Table 3.4, Table 3.5 and Table 3.6. For the ones whose mass values, spectral data and pattern matched, standards were provided. Finally, the spectral values and retention times were compared with the carotenoids in each chromatogram. However, some of the standards, especially cis-structures of many carotenoids are not available, tentative identification was made. Finally, system suitability and validation parameters for each carotenoid with the proposed LC methods were calculated as shown in Table 3.7 and Table 3.8 for the green microalgae and in Table 3.9 for the brown microalga.

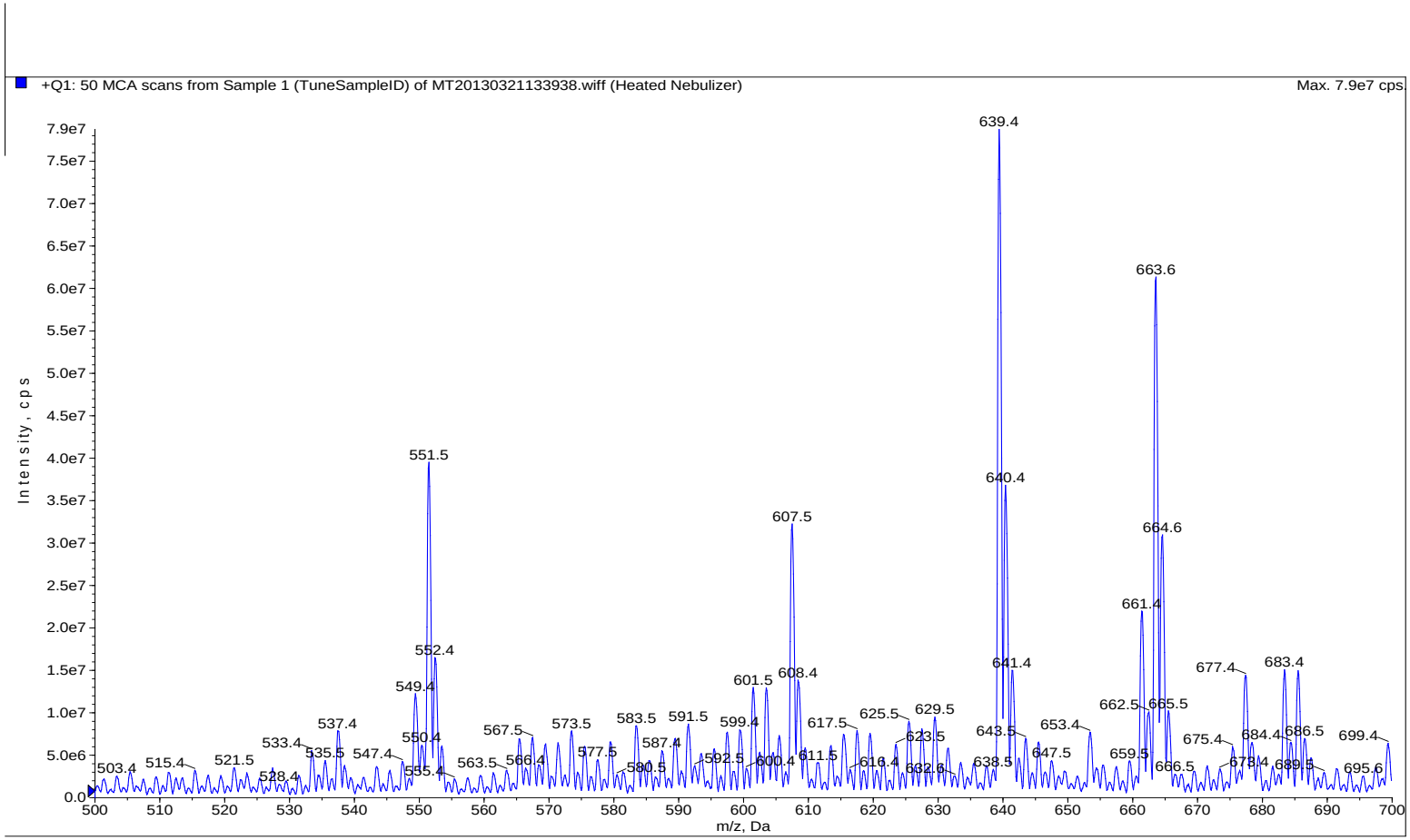


Figure 3.11. Mass spectra of *Prochloroccus sp.* extract

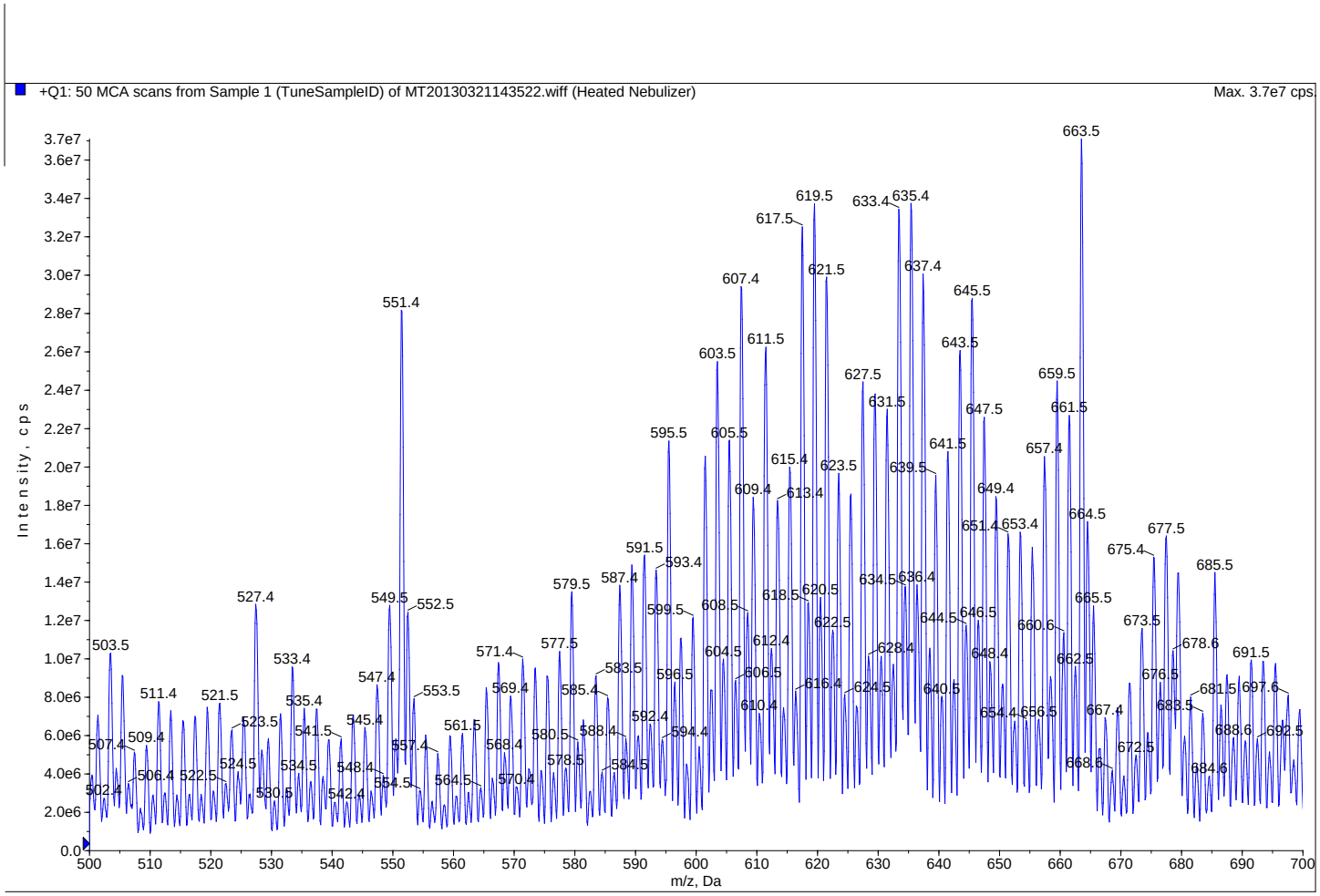


Figure 3.12. Mass spectra of *Scenedesmus protuberans* extract

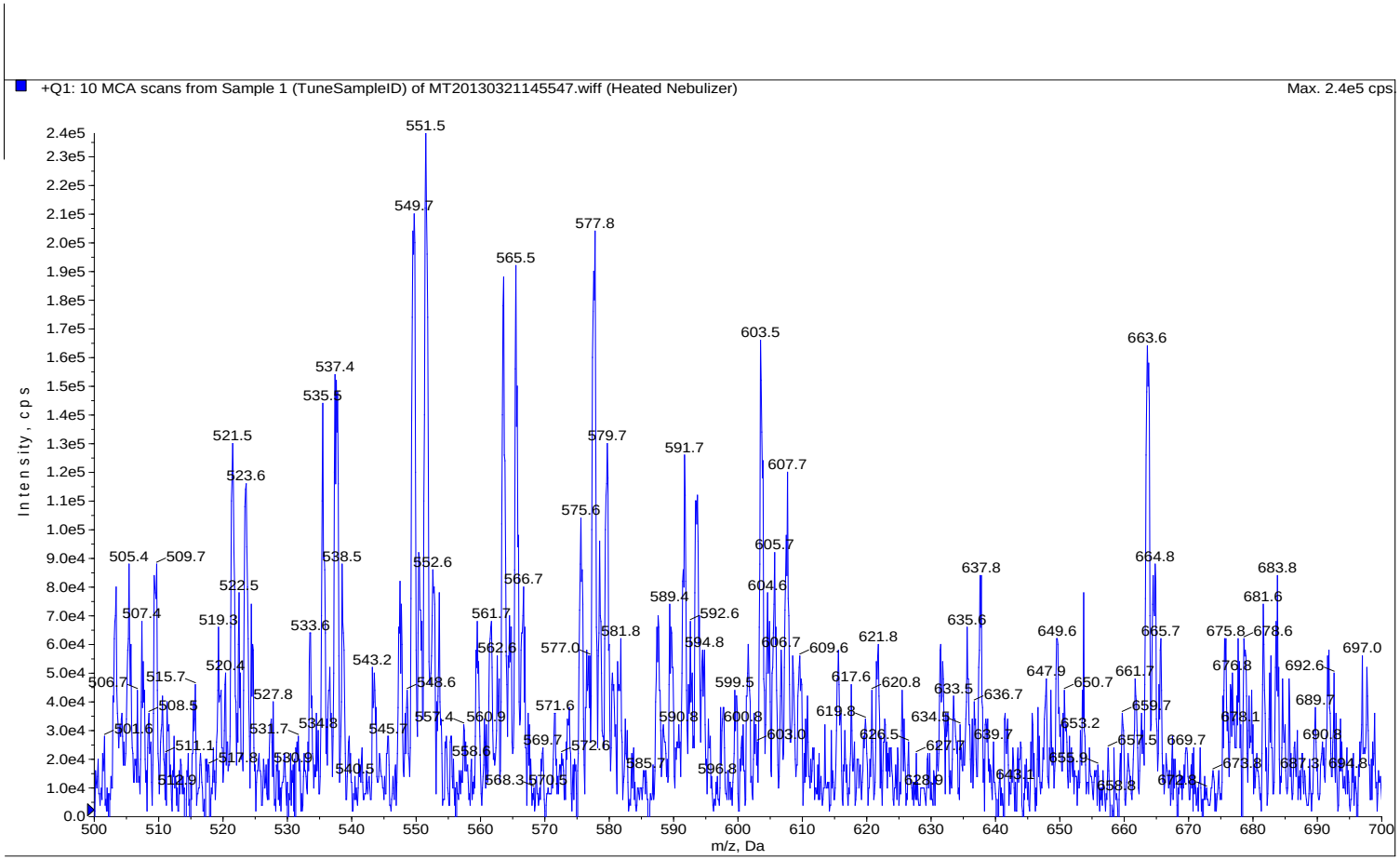


Figure 3.13. Mass spectra of *Nitzschia sp.* extract

Table 3.7. Summarized system suitability and validation parameters with proposed LC method (gradient elution with 70:25:5 MeOH:MTBE:H_2O at 450 nm, flow rate:1.0 mL/min.) for *Prochlorococcus sp.*

Compound	LOD (µg mL^{-1})	LOQ (µg mL^{-1})	r^2	Regression equation	Peak purity %	Capacity factor (k')	Selectivity factor(α)	Content (mg/g)
Neoxanthin	0.0106	0.035	0.9997	y=221.5x-103.67	99.3	1.43	1.87	0.45
Violaxanthin	0.0136	0.045	0.9998	y=128x-59.33	98.9	1.60	1.12	1.39
9-or 9'-cis-violaxanthin		no available standard			97.5	2.10	1.31	ND
Lutein	0.0032	0.011	0.9998	y=301.94x-26.27	99.5	3.11	1.49	2.54
9-or 9'-cis-lutein		no available standard			97.5	3.91	1.27	ND
α-carotene	0.0194	0.064	0.9997	y=696x-333.67	98.6	5.67	1.27	0.24
β-carotene	0.0221	0.073	0.9995	y=402.5x-194	99.1	5.99	1.06	0.3
9- or 9-cis-carotene	0.0297	0.098	0.9995	y=298.5x-144.33	97.6	6.25	1.04	0.13

Table 3.8. Summarized system suitability and validation parameters with proposed LC method (gradient elution with 70:25:5 MeOH:MTBE:H_2O at 450 nm, flow rate:1.0 mL/min.) for *Scenedesmus protuberans.*

Compound	LOD (µg mL^{-1})	LOQ (µg mL^{-1})	r^2	Regression equation	Peak purity %	Capacity factor (k')	Selectivity factor(α)	Content (mg/g)
Loroxanthin		no available standard			98.9	1.43	1.00	ND
Violaxanthin	0.0136	0.045	0.9998	y=128x-59.33	98.7	1.59	1.11	1.47
9-or 9'-cis-violaxanthin		no available standard			97.6	2.15	1.21	ND
Lutein	0.0032	0.011	0.9998	y=301.94x-26.27	99.5	3.15	1.47	2.45
9-or 9'-cis-lutein		no available standard			97.4	3.97	1.26	ND
α-carotene	0.0194	0.064	0.9997	y=696x-333.67	98.6	5.68	1.27	0.093
β-carotene	0.0221	0.073	0.9995	y=402.5x-194	99.2	6.05	1.08	0.55
9- or 9-cis-carotene	0.0297	0.098	0.9995	y=298.5x-144.33	97.4	6.25	1.03	0.19

Table 3.9. Summarized system suitability and validation parameters with proposed LC method (isocratic elution with 70:30-MeOH:ACN at 450 nm, flow rate:1.0 mL/min.) for *Nitzschia sp.*

Compound	LOD (μg mL^{-1})	LOQ (μg mL^{-1})	r^2	Regression equation	Peak purity %	Capacity factor (k')	Selectivity factor(α)	Content (mg/g)
Fucoxanthin	0.0017	0.0056	0.9998	y=181.02x+29.54	99.8	2.75	2.65	6.58
Fucoxanthinol	no available standard				96.1	2.90	1.05	ND
Neoxanthin	0.0185	0.061	0.9995	y=234.5x+56.12	97.5	4.38	1.59	0.94

3.5. Optimization of Solvent Extraction Conditions for Lutein

The importance of lutein was mentioned previously. Therefore, all of the solvent extraction conditions were optimized according to achieve the highest amount of lutein from the green microalgae.

Figure 3.14 shows the absorbance spectra for lutein and internal standard obtained by HPLC-DAD throughout the analysis. According to the data obtained lutein showed the maxiumum absorbance at 446.0 nm. Therefore all the HPLC analyses regarding the determination of lutein were performed at this wavelength. On the other hand, the internal standard maximum absorbance was recorded as 462.0 nm. Figure 3.15 demonstrates the HPLC chromatogram for the lutein standard and internal standard. It has been certainly seen that they are well separated form each other.

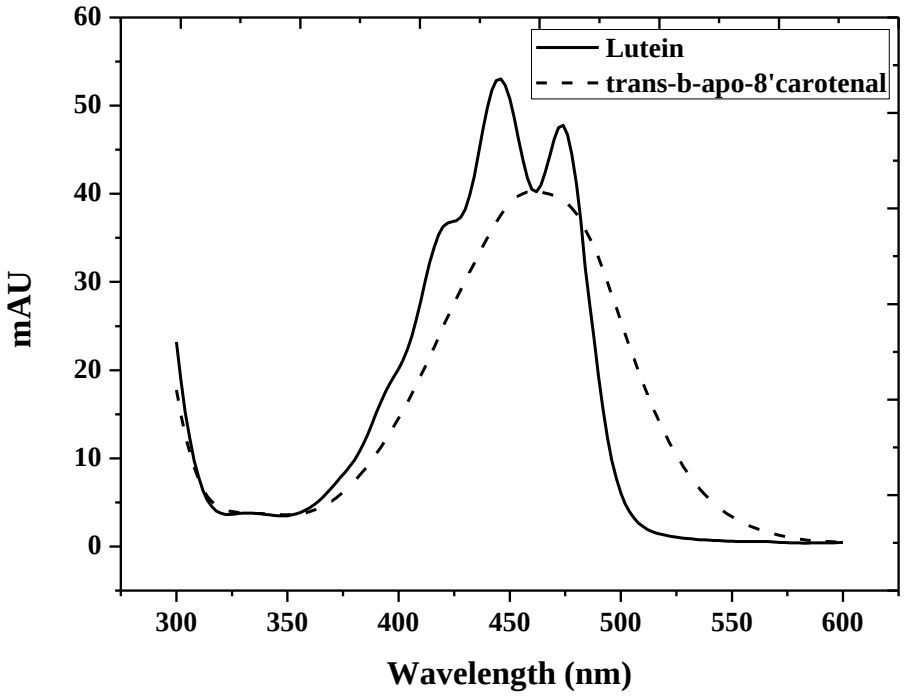


Figure 3.14 Absorbance spectra for lutein and trans-β-apo-8’-carotenal (internal standard)

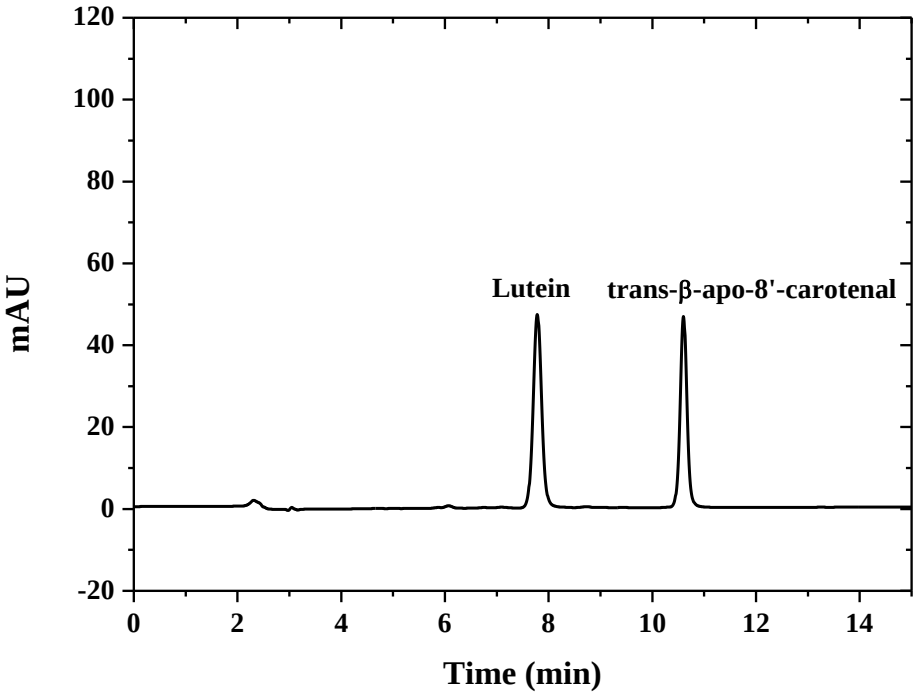


Figure 3.15. HPLC chromatogram for lutein and internal standard (trans-beta-apo-8'-carotenal) obtained by gradient elution (MeOH/MTBE/H_2O).

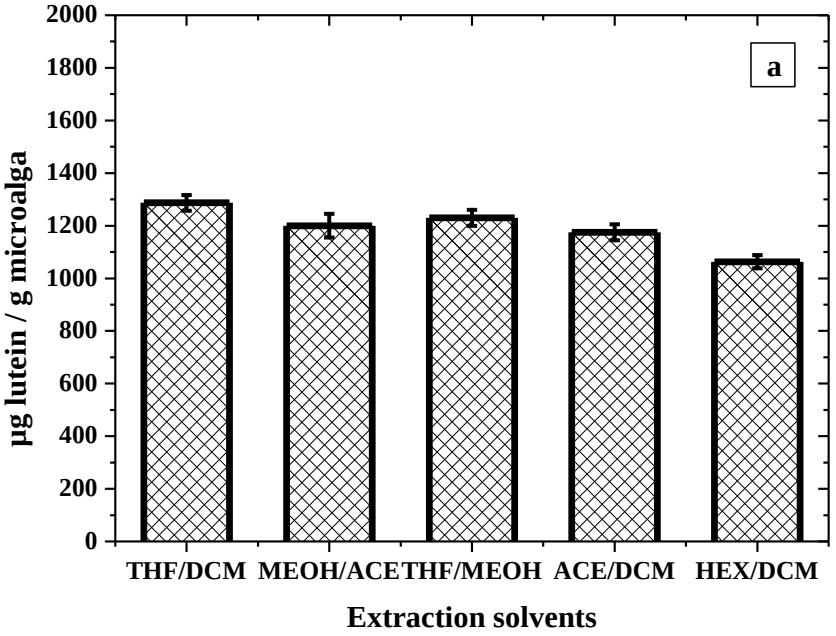
3.5.1. Effect of Solvent Combination on Lutein Extraction

The choice of the best solvent for extraction depends on the sample, its pre-treatment and the carotenoid composition. The adequate selection of the solvent plays an important role in increasing the efficiency of extraction. Carotenoids are extracted by using various types of solvents such as tetrahydrofurane, dichloromethane, methanol, ethanol, acetone, hexane or different combinations of them (Prasad 2011, Dias 2010 and Wang 2010). In this study, a suitable extraction procedure was tried to be developed for the extraction of carotenoids in two microalgae. Figure 3.16 shows the effect of solvent on lutein extraction from microalgae obtained when various binary organic solvent combinations (1:1) such as THF/DCM (tetrahydrofurane/dichloromethane), MEOH/ACE (methanol/acetone), THF/MEOH (tetrahydrofurane/methanol), ACE/DCM (acetone/dichloromethane) and HEX/DCM (hexane/dichloromethane).

According to the results obtained under the specified experimental conditions, THF/DCM showed the best binary composition for the extraction of lutein from both of the microalgae. This might be due to the excellent solubility of lutein in THF and DCM (Craft and Soares 1992). Since the extraction efficiency was affected by the solvent type and polarity, THF/DCM solvent combination was used as extraction solvent in the following experiments.

3.5.2. Effect of Saponification Time on Lutein Extraction

Saponification is mainly effective for removing colorless contaminating lipids and for destroying the chlorophyll. It also hydrolyzes carotenoid esters. Carotenols esterified with one or more fatty acids are encountered more frequently than are carotenoid carboxylic acid esters. Saponification of carotenol esters is advantegous before submission to HPLC. This has the advantage due to concentrating the carotenoids by removal of neutral lipids. In addition, it is also easier to characterize and determine quantitatively the free carotenols than the fatty acid esters that occur (Britton 1995a, Bechtold and Mussak 2009). For this reason, it is important to find out the optimum saponification time for each sample. Figure 3.17 shows the effect of saponification time on lutein extraction for both of the microalgae. The data clearly indicate that, two microalgae have different lutein content profiles. Our reserach suggests that lutein is mainly in free form in *Prochlorococcus sp.* as 5.0 min. saponification time is sufficient. On the other hand, it requires 2h-saponification time to reach the maximum lutein amount in *Scenedesmus protuberans* indicating that most of the lutein is in the form of carotenol esters. For both of the microalgae, HPLC analyses show that less amount of lutein has been observed if saponification has not been performed (data not shown).



a

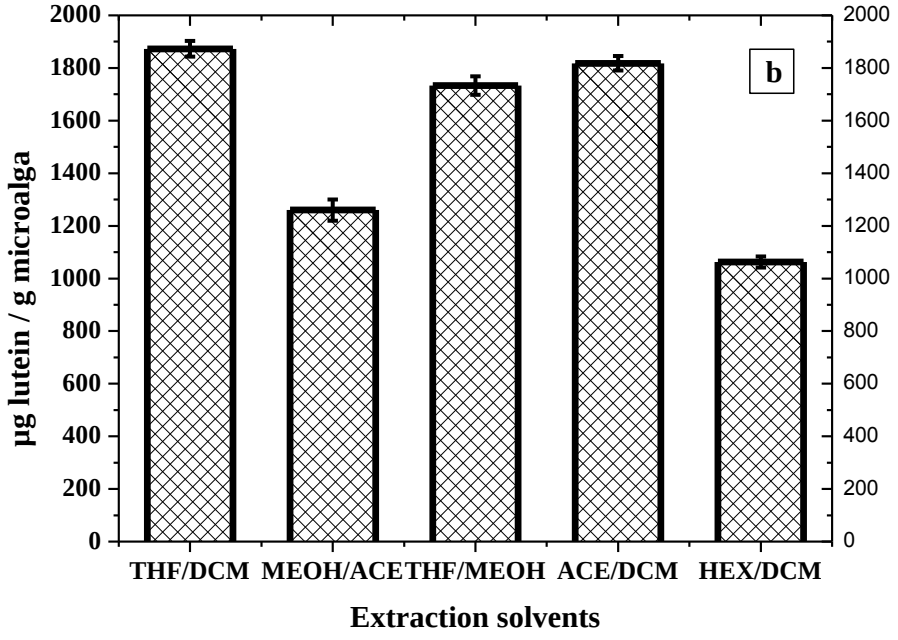


Figure 3.16. Effect of binary organic solvent combinations on extraction of lutein (a) *Prochlorococcus sp.* (b) *Scenedesmus protuberans.* Experimental conditions: 0.25 g microalga,10.0 mL solvent, 10.0 min. extraction x 3 times and 16.0 h saponification, 25 ^{0}C.

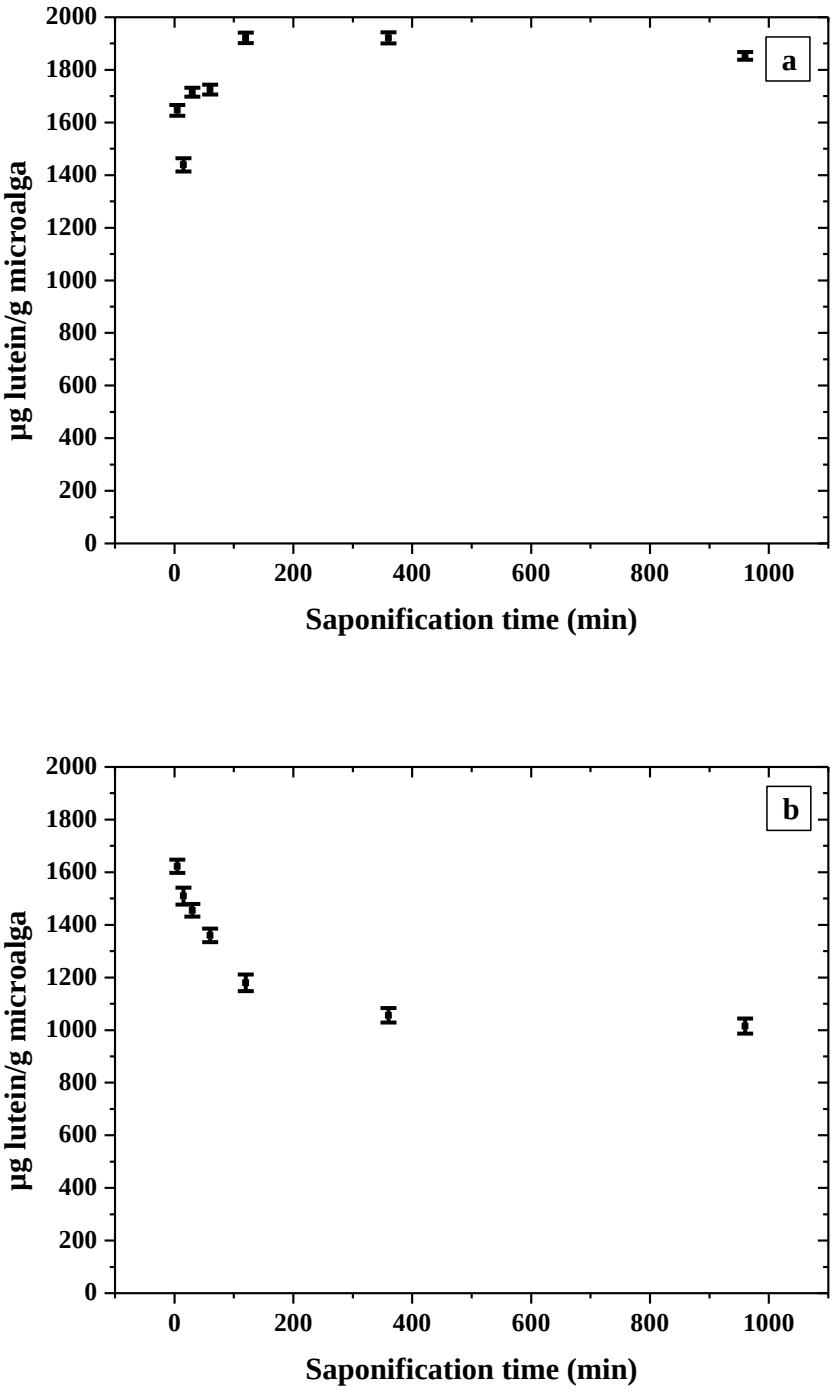


Figure 3.17. Effect of saponification time on extraction of lutein (a) *Prochlorococcus sp*. (b) *Scenedesmus protuberans.* Experimental conditions: 0.25 g microalga, 10.0 mL THF/DCM, 10.0 min. extraction x 3 times, 25 ^{0}C.

3.5.3. Effect of Contact Time on Lutein Extraction

The effect of contact time on lutein extraction for two microalgae was investigated by conducting the experiments in THF/DCM at 25.0^{o}C. In a liquid-solid extraction process, the following steps occur: (i) solvent transfer from the bulk of the solution to the surface of the solid; (ii) penetration or diffusion of the solvents into the pores of the solid; (iii) dissolution of the solute into the solvent; (iv) solute diffusion to the surface of the particle; and (v) solute transfer to the bulk of the solution. Any of the five basic steps may be responsible for limiting the extraction rate but in the operations that can occur because of the solubility of the solute or its miscibility with the solvent, the rate of the extraction is most likely to be controlled by the diffusion phenomena (Strati and Oreopoulou, 2011). As it was observed in Figure 3.18, lutein extraction depended on extraction time, showing a high initial rate of extraction that decrease with time until an almost equilibrium condition was reached. Based on the Figure 3.18, increase of the sonication time did not lead to significant variations on the concentrations of lutein. Therefore, 15-min extraction time seemed sufficient for the extraction of lutein for both of the microalgae.

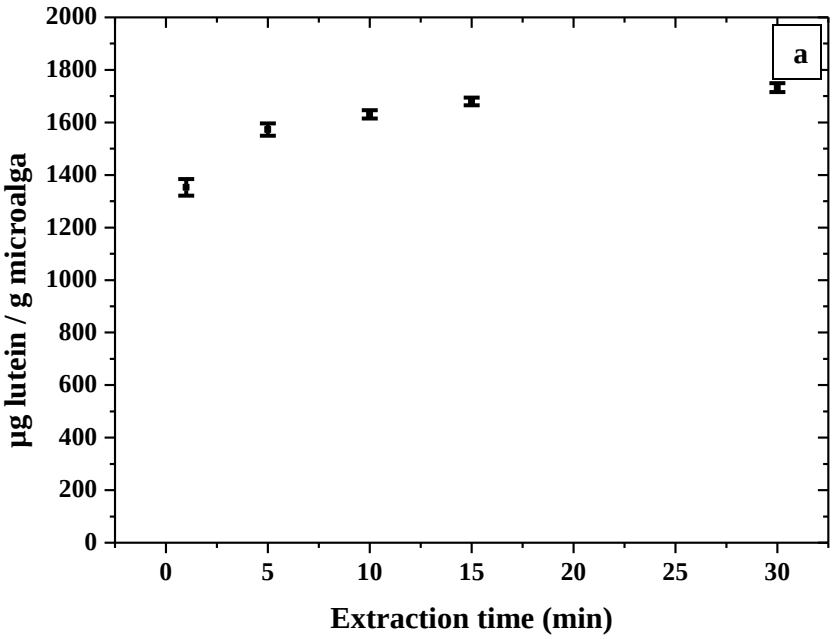
3.5.4. Effect of Temperature on Lutein Extraction

Generally, temperature has a positive effect on extraction efficiencies and rates when it is not too high, as some of the active components degrade with temperature. In order to study the effect of temperature on the extraction of lutein from two microalgae, extraction procedure was applied at 25, 40 and 60^{o}C and the results are showed as a function of time (Figure 3.19). Limiting factor for the choice of extraction temperature was the boiling point of the solvents used and the need to avoid undesirable reactions such as isomerization and/or oxidation of carotenoids. The degradation of lutein in the temprature range was not obsreved. The results indicate that with the increase of temperature, a small increase in the final extraction efficiency of lutein can be observed for *Scenedesmus protuberans*. However, the case is not the same for *Prochlorococcus sp.* Upon increase of temperature from 25 to 40 ^{0}C, no dramatic change was seen in lutein amount whereas there appeared a significant increase when temperature changed from 40 to 55^{o}C. At the

temperature examined, the improved extractibility of lutein with temperature increase in *Prochlorococcus sp.* is possibly related to the destruction of cellular structure and as a result of this fact, to the higher lutein content released from the microalgal matrix. Probably cell wall membrane gets ruptured due to high temperature allowing the liberation of pigments. Evidence from the experimental results showed that different morphology of the two microalgae affected their extraction profiles.

3.5.5. Effect of Number of Extractions on Lutein Extraction

To increase the extractability of lutein, succesive extractions were performed until no color was observed in the extract. For this purpose, ten extractions were done for each microalgae. Lutein recovery was significantly affected by the number of extractions as illustrated in Figure 3.20. The data indicate that little amount of lutein has been obtained after the sixth extraction from *Scenedesmus protuberans* while some lutein has been observed even after the nineth extraction from *Prochlorococcus sp.* In order to make sure that whether cells were disrupted or not, optical microscope images have been taken. According to those images it has been observed that *Scenedesmus protuberans* have completely ruptured while the case in *Prochlorococcus sp*. has not been the same. However it has been observed that the cells of *Prochlorococcus sp.* was sturdy and decoloration was observed within its cells when compared with the non-extracted cells (data not shown).



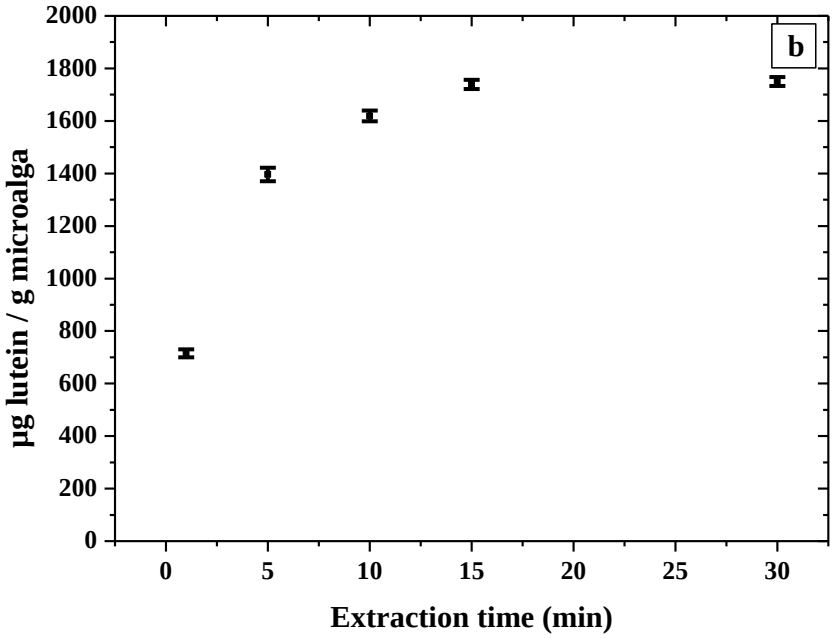


Figure 3.18. Effect of contact time on extraction of lutein (a) *Prochlorococcus sp*. (b) *Scenedesmus protuberans*. Experimental conditions:0.25 g microalga, 10.0 mL THF/DCM, 5.0 min. saponification for *Prochlorococcus sp*. and 2.0 h saponification for *Scenedesmus protuberans*, 25 ^{0}C.

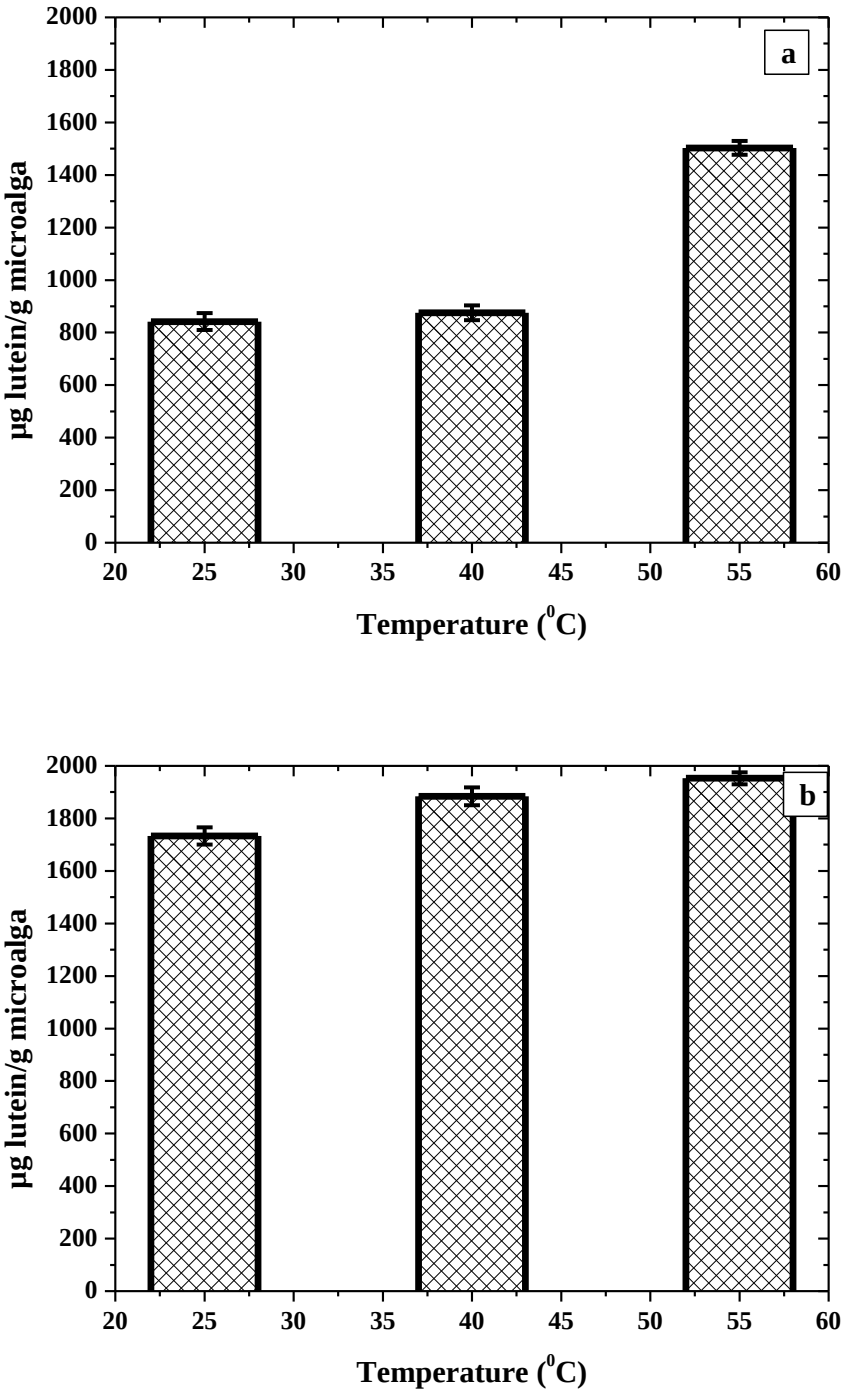


Figure 3.19. Effect of temperature on extraction of lutein (a) *Prochlorococcus sp*. (b) *Scenedesmus protuberans*. Experimental conditions: 0.25 g microalga, 10.0 mL THF/DCM, 15 min extraction x 3 times, 5.0 min. saponification for *Prochlorococcus sp*. and 2.0 h saponification for *Scenedesmus protuberans*, 25 ^{0}C.

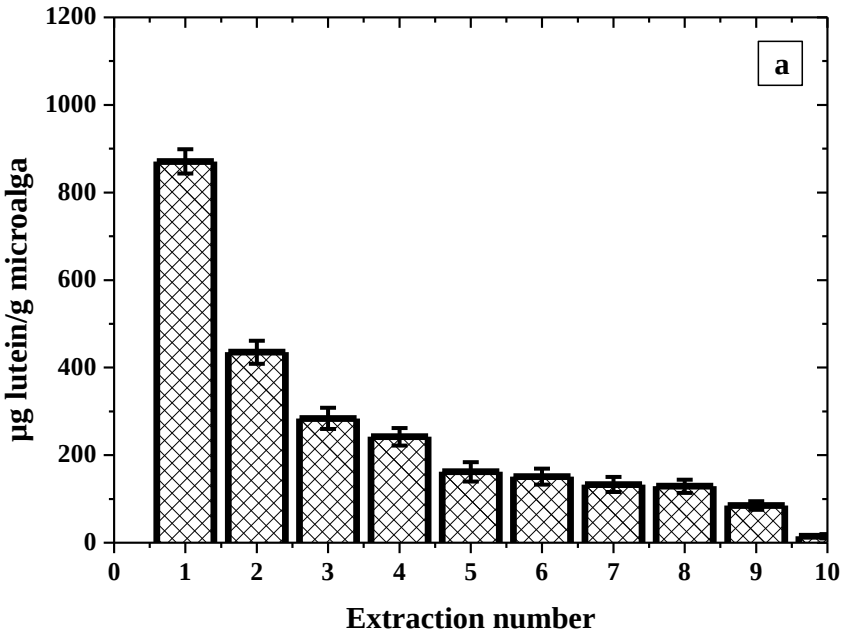


Figure 3.20. Effect of temperature on extraction of lutein (a) *Prochlorococcus sp.* (b) *Scenedesmus protuberans*. Experimental conditions: 0.20 g microalga, 10.0 mL THF/DCM, 15 min extraction x 10 times, 5.0 min. saponification for *Prochlorococcus sp*. and 2.0 h saponification for *Scenedesmus protuberans*.

3.6. Method Validation

Literature studies show that there are a few number of standard or certified reference materials for the method validation of carotenoids. Most of them are limited in terms of content and matrix (Kimura 2007 and Dias 2008) In addition, some of them are produced no longer. Since plant extract is thought to be the most relevant material with the microalgal extract, in the validation of our method with a certified reference material, BCR 485 (CRM-Mixed Vegetables) has been selected for the determination of some carotenoids. According to the analyses performed as designated in Table 3.10, the results obtained are consistent with CRM values of the carotenoids present in the sample.

Table 3.10. Method validation results for some carotenoids using BCR 485 (CRM-Mixed Vegetables)

Carotenoids	BCR 485 (mg/kg)	Method used (mg/kg)	
	Mixed vegetables	5-min saponification time	2-h saponification time
Lutein	12.5 ± 0.8	12.5 ± 0.1	12.6 ± 0.2
α-carotene	10.5 ± 0.6	10.5 ± 0.3	10.5 ± 0.5
β-carotene	23.7 ± 1.5	23.7 ± 0.6	23.7 ± 0.9

3.7. Improving the Lutein Content using Different Nitrogen Sources

A number of different nitrogen sources, such as ammonia, nitrate, nitrite and urea, can be used as nitrogen source for growing microalgae (Becker 2004). Among these, urea ($CO(NH_2)_2$) is a small-molecular weight polar and relatively lipid-insoluble organic compound which can be considered as a combined source of nitrogen and carbon. In literature, several examples can be found where urea is shown to be an effective combined source of N and C for the production of *S. Platensis, Neochloris oleoabundans* and *Chlorella sp.* under different cultivation modes (Li 2008, Becker 2004, Rangel-Yagui 2004, Sánchez-Luna 2004, Soletto 2005, Hsieh and Wu 2009).

This study aimed at assessing the effect of different nitrogen sources on biomass productivity and lutein. Table 3.11 and Table 3.12 show the growth rates and lutein accumulation of lutein grown on different N-sources for *Prochlorococcus sp.* and *Scenedesmus protuberans*, respectively. According to the results obtained, for an equivalent nitrogen concentration, urea gave higher yields for lutein accumulation. Some authors (Goldman 1977, Stengel and Soeder 1975) reported that ammonium was an excellent nitrogen source for marine as well as freshwater algae. On the other hand it has not been as common as nitrate for *Chlorella protothecoides* (Shi 2000) as a nitrogen source as in the case of microalgae used in this study which might be due to its inconvenience for sterilization and the lethal effect on cells due the severe drop in pH after ammonium has been consumed. Urea as a nitrogen source was found to be superior in several respects to the commonly used nitrogen source, nitrate.

On the other hand growth rates in cultures grown on urea were lower than the growth rates when other nitrogen sources are used. This might be due to the fact that urea is

an organic source while the rest are inorganic sources of nitrogen. Probably the accumulation of lutein due to utilization of organic source may not be same due to metabolic pathways when inorganic sources are used. In addition this organic compound can be considered as a combined source of nitrogen and carbon. Another reason why urea gave higher yields for an equivalent nitrogen concentration might be due to the fact that it is usually hydrolyzed into ammonia and bicarbonate and therefore leads smaller pH fluctuations in the medium during the algal growth (Perez-Garcia , 2011).

Table 3.11. Growth rates and lutein accumulation of *Prochlorococcus sp.* grown on different N-sources.

Nitrogen sources	**Log phase (day)**	**μ_{max} (day^{-1})**	**Lutein accumulated (mg/g)**
$NaNO_3$	4-8	0.110	2.54 ± 0.13
$NaNO_2$	4-8	0.376	1.82 ± 0.10
NH_4Cl	3-9	0.262	0.42 ± 0.11
CH_4N_2O	4-8	0.122	3.31 ± 0.17

Table 3.12. Growth rates and lutein accumulation of *Scenedesmus protuberans* grown on different N-sources.

Nitrogen sources	**Log phase (day)**	**μ_{max} (day^{-1})**	**Lutein accumulated (mg/g)**
$NaNO_3$	1-30	0.419	2.45 ± 0.16
$NaNO_2$	1-30	0.108	2.17 ± 0.20
NH_4Cl	1-11	0.173	0.70 ± 0.15
CH_4N_2O	1-30	0.080	3.86 ± 0.11

3.8. Improving the Lutein Content using Different Light Sources

Visible light is the one segment of the electromagnetic spectrum, as depicted in Figure 3.21. The various types of radiation differ in their wavelengths, and consequently in the amounts of energy carried by their individual quanta. Radiation of 750 nm and above has an energy content that is too low to mediate chemical changes; hence, radiant energy absorbed in this range will only appear as thermal effects.

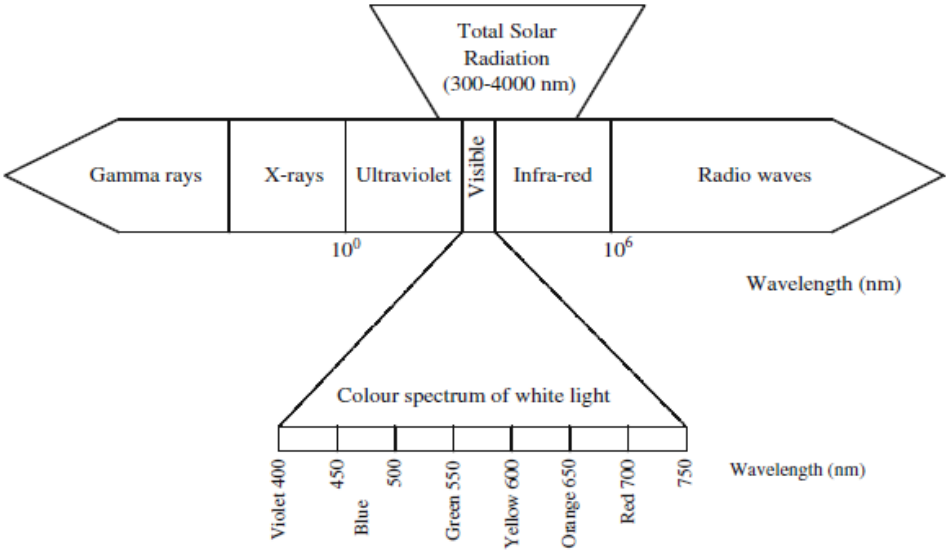


Figure 3.21. Whole electromagnetic spectrum, with detailed spectral pattern of visible light (Source: Carvalho 2011)

Conversely, radiation of 380 nm and below brings about ionizing effects. Between 380 and 750 nm, the energy content is sufficient to produce chemical changes in the absorbing molecules, as happens throughout the photosynthetic pathways prevailing in microalgae (Kommareddy and Anderson 2003). Hence, visible light is the main source of energy for autotrophic microalgae to produce organic compounds using the photosynthetic process. Energy absorption by photosynthetic organisms is thus dependent on the chemical nature of their constitutive pigments. The major pigment groups present in microalgae like chlorophylls, phycobilins, and carotenoids (carotenes and xanthophylls), are described in Table 3.13 (Masojidek 2004).

Within the major group mentioned, the most important molecule is chlorophyll a. Accessory pigments include chlorophylls b and c, as well as carotenoids; the former absorb other wavelengths of the radiation spectrum and pass their energy onto chlorophyll a, whereas the role of carotenoids seems to be absorption of excess light (and thus protection of chlorophyll integrity). Carotenoids are usually red, yellow or orange, so they do not absorb light in those regions, but instead in the violet/ blue and blue/green regions of visible light (Kommareddy and Anderson 2003). Phycobilins are water-soluble pigments,

consisting of many closely related compounds-e.g., phycocyanin, a blue pigment present in blue-green microalgae.

Table 3.13. Photonic features of major pigments in microalgae
(Source: Masojidek , 2004)

Pigment group	Color	Absorption bands (nm)		Pigments
Chlororphylls	Green	450-475 630-675	Hydrophobic	Chlorophyll a Chlorophyll b Chlorophyll c_1, c_2, d
Phycobilins	Blue, red	500-650	Hydrophilic	Phycocyanin Phycoerythrin Allophycocyanin
Carotenoids	Yellow, orange	400-550	Hydrophobic	β-Carotene α-Carotene Lutein Violaxanthin Fucoxanthin

When different light sources were used in order to increase the lutein content in green microalgae, the results were not as expected. It is obvious that putting the light sources in the bottles decrease the amount of lutein in all cases.

Table 3.14. Growth rates and lutein accumulation of *Prochlorococcus sp.*grown in the presence of light sources

Light source	Log phase (day)	μ_{max} (day^{-1})	Lutein accumulated (mg/g)
Control	4-8	0.117	2.57
Red	1-6	0.347	1.73
Blue	1-20	0.153	1.57
White	1-20	0.116	1.86

Table 3.15. Growth rates and lutein accumulation of *Scenedesmus protuberans* grown in the presence of light sources

Light source	Log phase (day)	μ_{max} (day^{-1})	Lutein accumulated (mg/g)
Control	1-20	0.410	2.48
Red	1-7	0.100	1.24
Blue	1-20	0.090	2.04
White	1-20	0.099	1.36

3.9. Improving the Lutein Content using Oxidative Stress Conditions

Carotenoids perform several functions in microalgae: they are involved in light harvesting, but also contribute to stabilize the structure and aid in the function of photosynthetic complexes-besides quenching chlorophyll triplet states, scavenging reactive oxygen species and dissipating excess energy (Demming-Adams 2002). The intrinsic antioxidant activity of carotenoids constitutes the basis for their protective action against oxidative stress; however, not all biological activities claimed for carotenoids relate to their ability to inactivate free radicals and reactive oxygen species.

Lutein synthesis is enhanced via addition of such chemicals as H_2O_2 and NaClO, which behave as inducers: in the presence of Fe^{2+}, they affect the redox state and generate stress-inducing chemical species. This induction of oxidative stress is expected because lutein holds a protection role due to its antioxidant features (Fernández-Sevilla 2010).

When compared to the effect of other oxidative sources, the addition of NaClO in the presence of Fe^{2+} the lutein accumulation in *Prochlorococcus sp.* can said to be remarkable as designated in Table 3.16. This might mean that it is accepted as a potential threat and thereby the microalga developed a protection mechanism inreasing its lutein content.

Table 3.16. Growth rates and lutein accumulation of *Prochlorococcus sp.* grown in the presence of oxidative sources

Oxidative stress sources in BBM medium	**Log phase (day)**	**μ_{max} (day^{-1})**	**Lutein accumulated (mg/g)**
no $\cdot OH$ and $^1O_2$	1-20	0.411	2.51 ± 0.17
0.1 mM H_2O_2 + 0.1 mM Fe^{2+}	1-20	0.137	1.92 ± 0.12
0.1 mM NaClO + 0.1 mM Fe^{2+}	1-20	0.147	4.53 ± 0.22
0.1 mM H_2O_2 + 0.1 mM NaOCl	1-20	0.170	2.92 ± 0.19

On the other hand, it does not seem to affect the metabolism of *Scenedesmus protuberans* positively in terms of lutein accumulation (Table 3.17). Probably the microalga could not overhelm any of these oxidative stress conditions.

Table 3.17. Growth rates and lutein accumulation of *Scenedesmus protuberans* grown in the presence of oxidative sources

Oxidative stress sources in BBM medium	**Log phase (day)**	**μ_{max} (day^{-1})**	**Lutein accumulated (mg/g)**
no $\cdot OH$ and $^1O_2$	1-30	0.116	2.47 ± 0.14
0.1 mM H_2O_2 + 0.1 mM Fe^{2+}	1-20	0.107	2.06 ± 0.17
0.1 mM NaClO + 0.1 mM Fe^{2+}	1-20	0.097	2.06 ± 0.20
0.1 mM H_2O_2 + 0.1 mM NaOCl	1-20	0.077	2.21 ± 0.13

3.10. Optimization of Solvent Extraction Conditions for Fucoxanthin

The absorbance spectra for fucoxanthin and astaxanthin (internal standard) obtained by HPLC-DAD throughout the analysis was shown in Figure 3.22. All the HPLC analyses performed at 450.0 nm. Figure 3.23 demonstrates the HPLC chromatogram for the fucoxanthin standard and astaxanthin (internal standard).

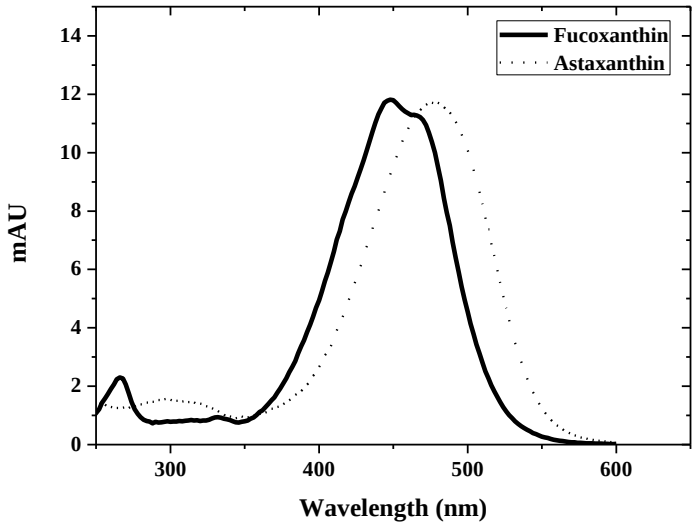


Figure 3.22. Absorbance spectra for fucoxanthin and astaxanthin (internal standard)

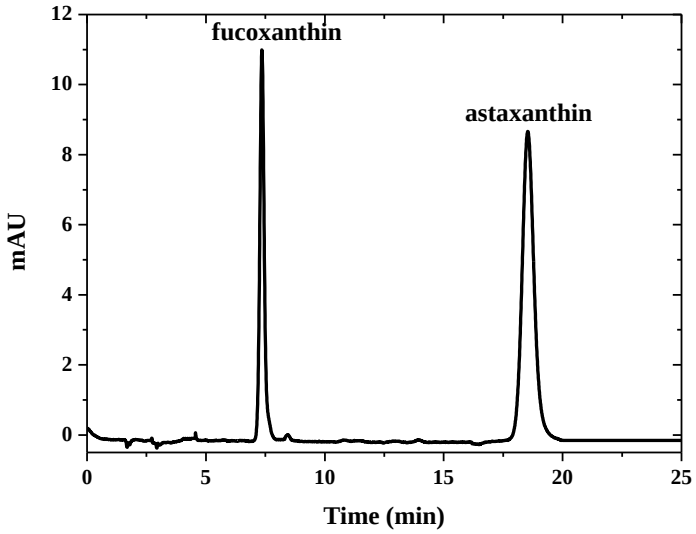


Figure 3.23. HPLC chromatogram for fucoxanthin and astaxanthin (internal standard) obtained at 450 nm (isocratic analysis,70:30-MeOH:ACN, flow rate: 1.0 mLmin^{-1})

3.10.1. Effect of Solvent Combination on Fucoxanthin Extraction

For the extraction of fucoxanthin, same solvent combinations (1:1) such as THF/DCM (tetrahydrofurane/dichloromethane), MEOH/ACE (methanol/acetone), THF/MEOH (tetrahydrofurane/methanol), ACE/DCM (acetone/dichloromethane) and HEX/DCM (hexane/dichloromethane). were applied. As in the case of green microalgae,

THF/DCM showed the best binary composition for the extraction of fucoxanthin from brown microalga as can be derived from Figure 3.24. This might be due to presence of high amount of fucoxanthin in the structure which is also highly polar carotenoid. THF/DCM solvent combination was used as extraction solvent combination in the following experiments regarding the extraction of fucoxanthin.

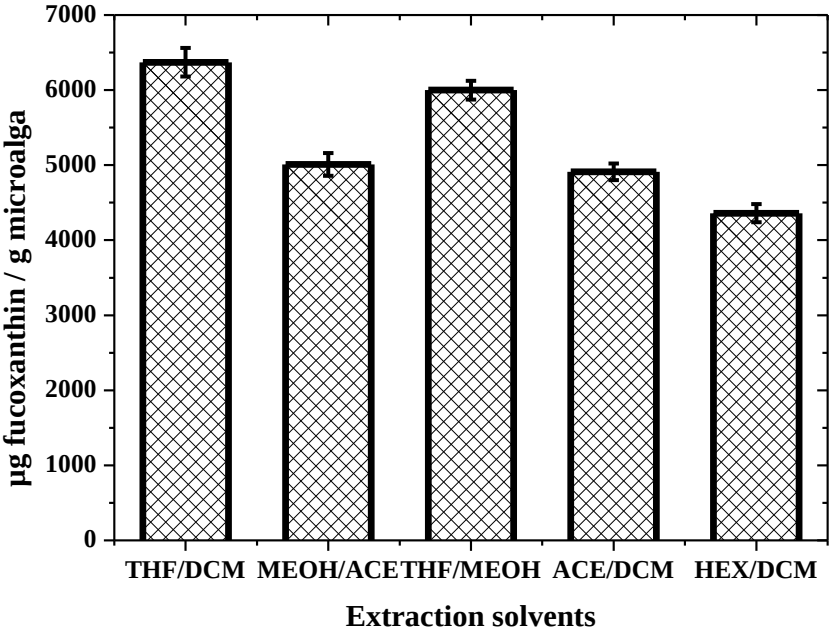


Figure 3.24. Effect of binary organic solvent combinations on extraction of fucoxanthin from Nitzschia sp. (Experimental conditions: 0.25 g microalga,10.0 mL solvent, 10.0 min. extraction x 3 times and 25 ^{0}C).

3.10.2. Effect of Contact Time on Fucoxanthin Extraction

The effect of contact time on fucoxanthin extraction from the microalga was investigated by conducting the experiments in THF:DCM (1:1) at 25.0^{o}C. As it was observed in Figure 3.25, fucoxanthin extraction depended on extraction time, showing a high initial rate of extraction that decrease with time until an almost equilibrium condition was reached. In addirion, the increase of the sonication time did not lead to significant variations on the concentrations of fucoxanthin. Therefore, 15-min extraction time seemed to be sufficient for the maximum extraction of fucoxanthin from the microalga.

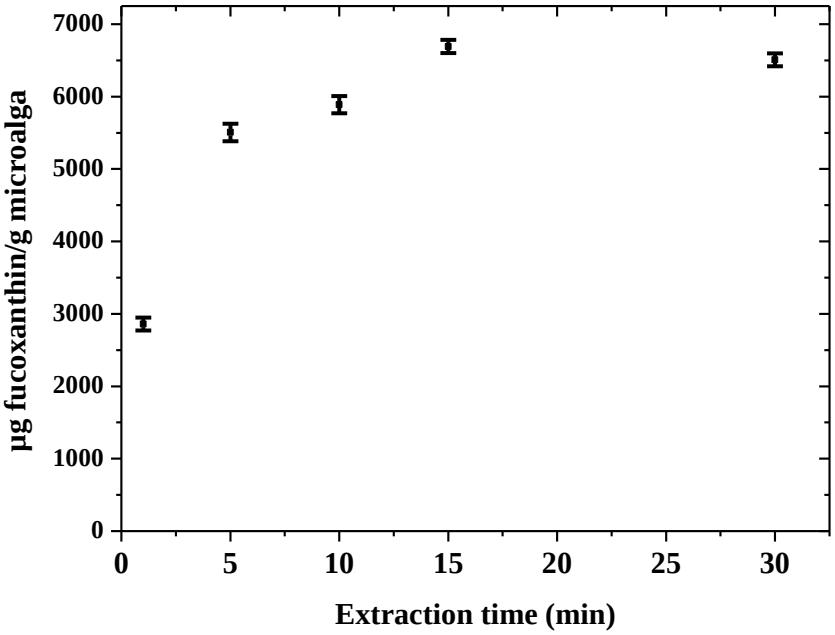


Figure 3.25. Effect of contact time on extraction of fucoxanthin from *Nitzschia sp.* (Experimental conditions: 0.25g microalga, 10.0 mL THF/DCM x 3 extractions, 25ºC).

3.10.3. Effect of Number of Extractions on Fucoxanthin Extraction

To increase the extractability of fucoxanthin successive extractions were performed until no color was observed. For this purpose, six extractions were done. Fucoxanthin recovery was significantly affected by the number of extractions as illustrated in Figure 3.26. The data indicate that 95% of fucoxanthin extraction is completed in the first extraction process. It has been observed that the cells of *Nitzschia sp.* was sturdy and significant decoloration was observed within its cells when compared with the non-extracted cells (data not shown) after the first extraction.

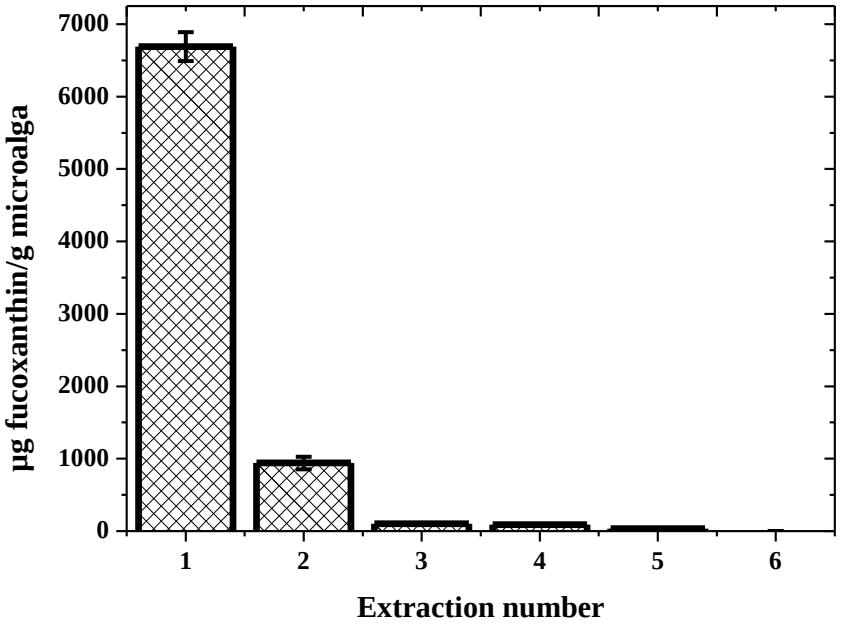


Figure 3.26. Effect of number of extractions on extraction of fucoxanthin from *Nitzschia sp.* (Experimental conditions: 0.25g microalga, 10.0 mL THF/DCM, 15 min, 25°C)

3.10.4. Effect of Temperature on Fucoxanthin Extraction

Generally, temperature has a positive effect on extraction efficiencies and rates when it is not too high, as some of the active components degrade with temperature. In order to study the effect of temperature on the extraction of fucoxanthin from microalga, extraction procedure was applied at 25, 40 and 55°C and the results are showed as a function of time (Figure 3.27). The results indicate that with the increase of temperature upto 40°C, a small increase in the final extraction efficiency of fucoxanthin can be observed for *Nitzschia sp.* Higher temperature probably allows the liberation of pigments due to increased kinetic energy and the contact between solvent molecules and the biomass. However, upon the increase of temperature to 55°C, the extraction of fucoxanthin decreases which might stem from undesirable reactions such as isomerization and/or oxidation of fucoxanthin.

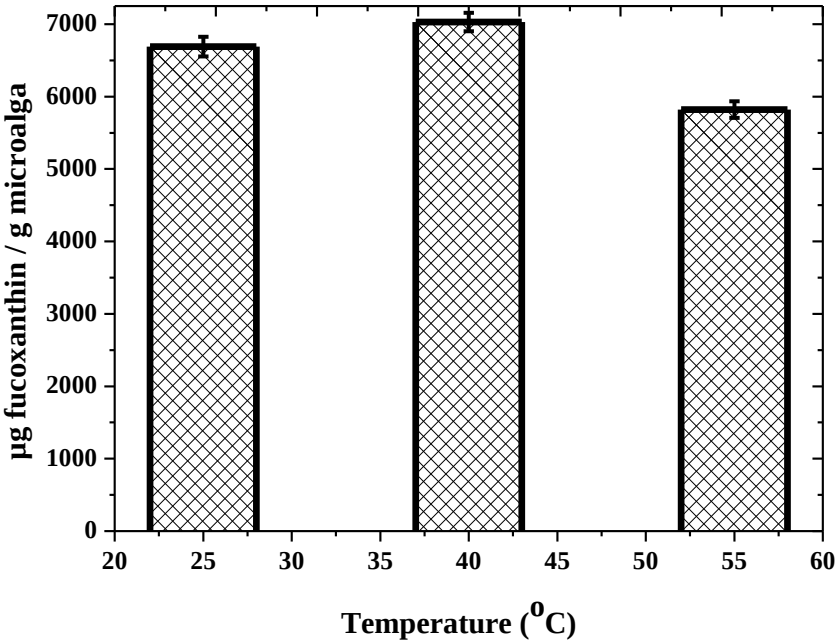


Figure 3.27. Effect of temperature on extraction of fucoxanthin from *Nitzschia sp.* (Experimental conditions: 0.25g microalga, 10.0 mL THF/DCM, 15 min. x 3)

3.11. Improving the Fucoxanthin Content using Different Nitrogen Sources

A number of different nitrogen sources, such as ammonia, nitrate, nitrite and urea, can be used as nitrogen source for growing microalgae (Becker 2004). Among these, urea ($CO(NH_2)_2$) is a small-molecular weight polar and relatively lipid-insoluble organic compound which can be considered as a combined source of nitrogen and carbon. In literature, several examples can be found where urea is shown to be an effective combined source of N and C for the production of *S. Platensis*, *Neochloris oleoabundans* and *Chlorella sp.* under different cultivation modes (Li 2008b, Becker 2004, Rangel-Yagui 2004, Sánchez-Luna 2004, Soletto 2005, Hsieh and Wu 2009).

This study aimed at assessing the effect of different nitrogen sources on biomass productivity and fucoxanthin accumulation of *Nitzschia sp*. Table 3.18 shows the growth rates and accumulation of fucoxanthin grown in the presence of different N-sources for *Nitzschia sp*. According to the results obtained, for an equivalent nitrogen concentration,

nitrite gave higher yields for fucoxanthin accumulation. It is yet an advantage that there appears an increase in fucoxanthin amount although microalga keeps its growth rate.

The reason why nitrite gave higher yields for an equivalent nitrogen concentration might be due to the fact that since there is no nitrate in the media to be converted to nitrite, the use of nitrite directly requires less energy. Hence it can make the process more spontaneous leading to the accumulation of higher amount of fucoxanthin (Perez-Garcia 2011).

Table 3.18. Growth rates and fucoxanthin accumulation of *Nitzschia sp.*grown in the presence of different N-sources.

Nitrogen sources	**Log phase (day)**	**μ_{max} (day^{-1})**	**Fucoxanthin accumulated (mg/g)**
$NaNO_3$	1-17	0.134	6.58 ± 0.14
$NaNO_2$	1-20	0.134	9.85 ± 0.19
NH_4Cl	1-17	0.167	3.74 ± 0.11
CH_4N_2O	1-20	0.141	3.35 ± 0.10

3.12. Improving the Fucoxanthin Content using Oxidative Stress Conditions

When compared to the effect of H_2O_2 plus Fe^{2+} and H_2O_2 plus NaClO, the addition of NaClO in the presence of Fe^{2+} the fucoxanthin accumulation in *Nitzschia sp.* can said to be remarkable. In the presence of these reactive oxygen species (ROS), the antioxidative carotenoids might be produced in order to protect the cells against oxidative damage.

Unfortunatley little information was known about the mechanism of the stimulating formation of fucoxanthin by ROS. However, the enhancement in the fucoxanthin accumulation using suitable concentrations of ROS in *Nitzschia sp.* was confirmed through this study.

Hydrogen peroxide is a product of microalgae via its oxidative metabolism. Nearly all living things decompose low concentrations of hydrogen peroxide to water and oxygen.

However, H_2O_2 can injure cells at high concentrations or lead to acclimation at moderate levels. Particularly in the presence of iron, hydrogen peroxide decomposes and generates the highly reactive hydroxyl radical through Fenton reaction. Moreover, the uncontrolled production of reactive oxygen species (ROS) molecules may destroy proteins, lipids and carotenoids. As with many other organisms, microalagae develop defence mechanisms against high levels of ROS (Wei 2008).

Table 3.19. Growth rates and fucoxanthin accumulation of *Nitzschia sp.*grown in the presence of oxidative sources

Oxidative stress sources in F/2 medium	**Log phase (day)**	**μ_{max} (day^{-1})**	**Fucoxanthin accumulated (mg/g)**
no $\cdot OH$ and $^1O_2$	1-17	0.136	6.58 ± 0.17
0.1 mM H_2O_2 + 0.1 mM Fe^{2+}	1-17	0.171	6.62 ± 0.12
0.1 mM NaClO + 0.1 mM Fe^{2+}	1-20	0.118	10.19 ± 0.22
0.1 mM H_2O_2 + 0.1 mM NaOCl	1-17	0.178	7.79 ± 0.19

According to the oxidative stress experimental results shown in Table 3.19, NaOCl plus Fe^{2+} stimulates the production of fucoxanthin better than the other oxidative stress sources. Probably, the microalga could more easily overcome the effect of H_2O_2 plus Fe^{2+}. However, the case may not be similar in other conditions. As the fucoxanthin is the main carotenoid that is highly accumulated in *Nitzschia sp.*,the increase in its amount due to the presence of NaClO plus Fe^{2+} might be regarded as a real stress condition that cannot be overhelmed as in the case of H_2O_2 plus Fe^{2+}.

3.13. Improving the Fucoxanthin Amount using Different Light Sources

In the presence of different light sources, it wa sobserved that none of these light sources were successful in increasing the fucoxanthin amount in *Nitzschia sp.* It is interesting that brown microoalga responded positively to the red light in terms of growth rate and log phase is almost halved (Table 3.20) This means that in twenty days, more

biomass and more fucoxanthin can be obtained. Therefore, red light stimulates the growth of brown microalga causeing more biomass to be obtained.

In order to increase microalgal biomass and specific metabolite productivity, long-term strategies of research in the field should encompass design and development of innovative PBRs, coupled with genetic engineering of strains. To attain maximum productivity in said PBRs, several parameters are to be accurately controlled; however, none of the many reactor configurations built to date is able to effectively handle all those parameters simultaneously.

Table 3.20. Growth rates and fucoxanthin accumulation of *Nitzschia sp.* grown in the presence of light sources

Light source	**Log phase (day)**	**μ_{max} (day^{-1})**	**Fucoxanthin accumulated (mg/g)**
Control	1-18	0.139	6.60 ± 0.12
Red	1-10	0.316	5.97 ± 0.16
Blue	1-12	0.260	5.49 ± 0.19
White	1-12	0.238	5.67 ± 0.17

CHAPTER 4

CONCLUSION

In recent years, there has been particular emphasis on obtaining more accurate data on the types and concentrations of carotenoids in foods for various health and nutrition activities. On the other hand, significant attention has recently been drawn to the use of microalgae since they produce carotenes and xanthophylls in rich amounts during their normal growth or when exposed to stress conditions. For this reason, carotenoid production has become one of the most successful branch of biotechnology of microalgae. For this purpose it has been expected to find the carotenoid-rich algal biomass and purified products. The analysis of carotenoids is complicated because of the diversity. Although all carotenoids contain conjugated double bonds, individual carotenoids differ in their antioxidant potential in humans. Therefore, new carotenoid sources are required to be investigated.

Another problem associated with analysis of carotenoids is the difficulty in obtaining standard compounds. At the end of this study, it is expected to have pure carotenoids from microalgae in high amounts. In addition, it can be said that carotenoids have become an important subject of study due to the prevention of several lifestyle-related diseases. Therefore, research into their physiological functions and their use as dietary markers requires sensitive, accurate, precise and efficient measurement. Further advances in these methodological areas will contribute to basic, clinical and public health research projects dealing with prevention and management of chronic disease through nutritional intervention. Another critical stage in this study is the sample pre-treatment and cultivation conditions of algal materials since carotenoids are highly sensitive to light, temperature and oxidation. Therefore, the techniques used are important stages and thus their optimization are required.

In conclusion, the future of microalgal biotechnology for the industrial carotenoid production seems, therefore, very promising and products are expected to develop in the following years.

REFERENCES

Astorg, P. 1997. Food Carotenoids and Cancer Prevention: An Overview of Current Research. *Trends in Food Science & Technology*. 8 (12):406-413.

Bechtold, T. and R. Mussak. 2009. *Handbook of Natural Colorants*, John Wiley & Sons, New York.

Becker, E. W. 1993. *Microalgae: Biotechnology and Microbiology*. Vol. 10: Cambridge University Press.

Bendich, A., and J. A. Olson. 1989. Biological Actions of Carotenoids. *Faseb Journal*. 3 (8):1927-1932.

Blanco, A. M., J. Moreno, J. A. Del Campo, J. Rivas, and M. G. Guerrero. 2007. Outdoor Cultivation of Lutein-Rich Cells of *Muriellopsis sp*. in Open Ponds. *Applied microbiology and biotechnology*. 73 (6):1259-1266.

Britton, G. 1991 Carotenoids. *Methods Plant Biochemistry*. 7:473-518.

Britton, G., S. Liaaen-Jensen, H. Pfander. 1995. *Carotenoids*. Birkhauser Verlag, vol.1A, 210-214, Basel.

Britton, G., S. Liaaen-Jensen, H. Pfander. 1996. *Carotenoids*. Birkhauser Verlag, vol.1B, 13-62, Basel.

Bushway, R. J. 1985. Separation of Carotenoids in Fruits and Vegetables by High-Performance Liquid-Chromatography. *Journal of Liquid Chromatography*. 8 (8):1527-1547.

Carvalho, A. P., S. O. Silva, J. M. Baptista, and F. X. Malcata. 2011. Light Requirements in Microalgal Photobioreactors: An Overview of Biophotonic Aspects. *Applied Microbiology and Biotechnology*. 89 (5):1275-1288.

Chiu, C. J., and A. Taylor. 2007. Nutritional Antioxidants and Age-Related Cataract and Maculopathy. *Experimental Eye Research*. 84 (2):229-245.

Craft, N. E., and J. H. Soares. 1992. Relative Solubility, Stability, and Absorptivity of Lutein and Beta-Carotene in Organic-Solvents. *Journal of Agricultural and Food Chemistry*. 40 (3):431-434.

Craft, N. E., and J. H. Soares. 1992. Relative Solubility, Stability, and Absorptivity of Lutein And. Beta.-Carotene in Organic Solvents. *Journal of Agricultural and Food Chemistry*. 40 (3):431-434.

Davies, B.H. 1976. “Carotenoids” In *Chemistry and Biochemistry of Plant Pigments*, Vol. 2, Goodwin, T.W. (Ed.), Academic Press, London, New York, San Francisco. 38–165.

Dawson, M.I. and P.D. Hobbs. 1994. “Synthetic chemistry of retinoids” In *Retinoids*, Sporn, M.B. Eds., Raven Press, New York, 5.

De Ritter, A.E. 1981. In Carotenoids as Colorants and Vitamin A Precursors, Academic Press, New York.

De Rosso, V. V., and A. Z. Mercadante. 2007. Identification and Quantification of Carotenoids, by HPLC-PDA-MS/MS, from Amazonian Fruits. *Journal of Agricultural and Food Chemistry*. 55 (13):5062-5072.

Del Campo, J. A., H. Rodríguez, J. Moreno, M. Vargas, J. n. Rivas, and M. G. Guerrero. 2001. Lutein Production by *Muriellopsis sp.* in an Outdoor Tubular *Photobioreactor*. *Journal of Biotechnology*. 85 (3):289-295.

Del Campo, J. A., J. Moreno, H. Rodríguez, M. Angeles Vargas, J. n. Rivas, and M. G. Guerrero. 2000. Carotenoid Content of Chlorophycean Microalgae: Factors Determining Lutein Accumulation in *Muriellopsis sp.* (Chlorophyta). *Journal of Biotechnology*. 76 (1):51-59.

Delgado-Vargas, F., A. R. Jimenez, and O. Paredes-Lopez. 2000. Natural Pigments: Carotenoids, Anthocyanins, and Betalains - Characteristics, Biosynthesis, Processing, and Stability. *Critical Reviews in Food Science and Nutrition*. 40 (3):173-289.

Demmig-Adams, B., and W. W. Adams. 2002. Antioxidants in Photosynthesis and Human Nutrition. *Science*. 298 (5601):2149-2153.

Dias, M. G., L. Oliveira, M. Camões, B. Nunes, P. Versloot, and P. J. Hulshof. 2010. Critical Assessment of Three High Performance Liquid Chromatography Analytical Methods for Food Carotenoid Quantification. *Journal of Chromatography A*. 1217 (21):3494-3502.

Everett, S. A., M. F. Dennis, K. B. Patel, S. Maddix, S. C. Kundu, and R. L. Willson. 1996. Scavenging of Nitrogen Dioxide, Thiyl, and Sulfonyl Free Radicals by the Nutritional Antioxidant Beta-Carotene. *Journal of Biological Chemistry*. 271 (8):3988-3994.

Falconer, M., E., M. J. Fishwick and D. G. Land D. 1964. Carotene oxidation and off-flavor development in dehydrated carrot. *Journal of the Science of Food and Agriculture*. 15, 857, 1964.

Fernández-Sevilla, J. M., F. A. Fernández, and E. M. Grima. 2010. Biotechnological Production of Lutein and Its Applications. *Applied Microbiology and Biotechnology*. 86 (1):27-40.

Frickel, F. 1984. "Chemistry and Physical Properties of Retinoids" In the *Retinoids,* Vol. 1, Sporn, M.B. Eds. Academic Press, Orlando. 7.

Fung, A., N. Hamid, and J. Lu. 2013. Fucoxanthin Content and Antioxidant Properties of *Undaria Pinnatifida. Food Chemistry*. 136 (2):1055-1062.

Goldman, J. C. 1977. Biomass Production in Mass Cultures of Marine Phytoplankton at Varying Temperatures. *Journal of Experimental Marine Biology and Ecology*. 27 (2):161-169.

Granado, F., B. Olmedilla, and I. Blanco. 2003. Nutritional and Clinical Relevance of Lutein in Human Health. *British Journal of Nutrition*. 90 (3):487-502.

Haila, K. M., B. R. Nielsen, M. I. Heinonen, and L. H. Skibsted. 1997. Carotenoid Reaction with Free Radicals in Acetone and Toluene at Different Oxygen Partial Pressures. *Zeitschrift Fur Lebensmittel-Untersuchung Und-Forschung a-Food Research and Technology*. 204 (2):81-87.

Hao, Z., B. Parker, M. Knapp, and L. L. Yu. 2005. Simultaneous Quantification of α-Tocopherol and Four Major Carotenoids in Botanical Materials by Normal Phase Liquid Chromatography–Atmospheric Pressure Chemical Ionization-Tandem Mass Spectrometry. *Journal of Chromatography A*. 1094 (1):83-90.

Hart, D. J., and K. J. Scott. 1995. Development and Evaluation of a Method for the Analysis of Carotenoids in Foods, and the Measurement of the Carotenoid Content of Vegetables and Fruits Commonly Consumed in the UK. *Food Chemistry*. 54 (1):101-111.

Heber, D., and Q. Y. Lu. 2002. Overview of Mechanisms of Action of Lycopene. *Experimental Biology and Medicine*. 227 (10):920-923.

Hsieh, C.-H., and W.-T. Wu. 2009. Cultivation of Microalgae for Oil Production with a Cultivation Strategy of Urea Limitation. *Bioresource Technology*. 100 (17):3921-3926.

Hurst, W.J., 2008. Methods of Analysis for Functional Foods and Nutraceuticals. CRC Press, USA.

Ip, P.-F., and F. Chen. 2005. Employment of Reactive Oxygen Species to Enhance Astaxanthin Formation in *Chlorella zofingiensis* in Heterotrophic Culture. *Process Biochemistry*. 40 (11):3491-3496.

Isler, H. and Gutmann, U.S., Carotenoids, Birkhauser Verlag, Basel, 1971.

Jaswir, I., D. Noviendri, H. M. Salleh, M. Taher, and K. Miyashita. 2011. Isolation of Fucoxanthin and Fatty Acids Analysis of *Padina Australis* and Cytotoxic Effect of Fucoxanthin on Human Lung Cancer (H1299) Cell Lines. *African Journal of Biotechnology*. 10 (81):18855-18862.

Jin, E., J. E. Polle, H. Lee, S. Hyun, and M. Chang. 2003. Xanthophylls in Microalgae: From Biosynthesis to Biotechnological Mass Production and Application. *Journal of Microbiology and Biotechnology*. 13 (2):165-174.

Kimura, M., and D. B. Rodriguez-Amaya. 1999. Sources of Errors in the Quantitative Analysis of Food Carotenoids by. *Archivos Latinoamericanos De Nutricion*. 49 (3):58S-66S.

Kimura, M., and D. B. Rodriguez-Amaya. 1999. Sources of Errors in the Quantitative Analysis of Food Carotenoids by. *Archivos Latinoamericanos De Nutricion*. 49 (3):58S-66S.

Kimura, M., C. N. Kobori, D. B. Rodriguez-Amaya, and P. Nestel. 2007. Screening and Methods for Carotenoids in Sweetpotato, Cassava and Maize for Plant Breeding Trials. *Food Chemistry*. 100 (4):1734-1746.

Klaui, H., Bauernfeind, J.C. (1981). "Carotenoids as Food Colors". In *Carotenoids as Colorants and Vitamin A Precursors* (J.C.Bauernfeind, ed.), 47-317, Academic Press, New York.

Kommareddy, A., and G. Anderson. 2003. Study of Light as a Parameter in the Growth of Algae in a Photo-Bio Reactor (PBR). Paper read at ASAE Annual International Meeting Presentation.

Kommareddy, A., and G. Anderson. 2003. Study of Light as a Parameter in the Growth of Algae in a Photo-Bio Reactor (PBR). Paper read at ASAE Annual International Meeting Presentation.

Krinsky, N. I., J. T. Landrum, and R. A. Bone. 2003. Biologic Mechanisms of the Protective Role of Lutein and Zeaxanthin in the Eye. *Annual Review of Nutrition*. 23:171-201.

Kurz, C., R. Carle, and A. Schieber. 2008. HPLC-DAD-MS Characterisation of Carotenoids from Apricots and Pumpkins for the Evaluation of Fruit Product Authenticity. *Food Chemistry*. 110 (2):522-530.

Ladislav, F., P. Vera, S. Karel, and V. Karel. 2005. Reliability of Carotenoid Analyses: A Review. *Current Analytical Chemistry*. 1 (1):93-102.

Le Marchand, L., J. H. Hankin, L. N. Kolonel, G. R. Beecher, L. R. Wilkens, and L. P. Zhao. 1993. Intake of Specific Carotenoids and Lung Cancer Risk. *Cancer Epidemiol.Biomarkers* Prev. 2: 183-187.

Lesellier, E., C. Marty, C. Berset, and A. Tchapla. 1989. Optimization of the Isocratic Non-Aqueous Reverse Phase (NARP) Separation of trans-cis alpha-Carotenes and beta-Carotenes. *HRC-Journal of High Resolution Chromatography*. 12 (7):447-454.

Li, Y., M. Horsman, B. Wang, N. Wu, and C. Q. Lan. 2008. Effects of Nitrogen Sources on Cell Growth and Lipid Accumulation of Green Alga *Neochloris oleoabundans*. *Applied Microbiology and Biotechnology*. 81 (4):629-636.

Maeda, H., M. Hosokawa, T. Sashima, K. Funayama, and K. Miyashita. 2005. Fucoxanthin from Edible Seaweed, *Undaria pinnatifida*, Shows Antiobesity Effect through Ucp1 Expression in White Adipose Tissues. *Biochemical and Biophysical Research Communications*. 332 (2):392-397.

Masojıdek, J., M. Koblızek, and G. Torzillo. 2004. 2 Photosynthesis in Microalgae. *Handbook of Microalgal Culture: Biotechnology and Applied Phycology*: 20.

Melendez-Martinez, A. J., I. M. Vicario, and F. J. Heredia. 2003. A Routine High-Performance Liquid Chromatography Method for Carotenoid Determination in Ultra-frozen Orange Juices. *Journal of Agricultural and Food Chemistry*. 51 (15):4219-4224.

Mise, T., M. Ueda, and T. Yasumoto. 2011. Production of Fucoxanthin-Rich Powder from *Cladosiphon okamuranus*. *Advance Journal of Food Science and Technology*. 3 (1):73-76.

Nguyen, M. L., and S. J. Schwartz. 1998. Lycopene Stability during Food Processing. *Proceedings of the Society for Experimental Biology and Medicine*. 218 (2):101-105.

Nomura, T., M. Kikuchi, A. Kubodera, and Y. Kawakami. 1997. Proton-Donative Antioxidant Activity of Fucoxanthin with 1, 1-Diphenyl-2-Picrylhydrazyl (DPPH). *IUBMB Life*. 42 (2):361-370.

Peng, J., J.-P. Yuan, C.-F. Wu, and J.-H. Wang. 2011. Fucoxanthin, a Marine Carotenoid Present in Brown Seaweeds and Diatoms: Metabolism and Bioactivities Relevant to Human Health. *Marine Drugs*. 9 (10):1806-1828.

Perez-Garcia, O., F. M. Escalante, L. E. de-Bashan, and Y. Bashan. 2011. Heterotrophic Cultures of Microalgae: Metabolism and Potential Products. *Water Research*. 45 (1):11-36.

Pfander, H., R. Riesen, and U. Niggli. 1994. and SFC of Carotenoids - Scope and Limitations. *Pure and Applied Chemistry*. 66 (5):947-954.

Pomeranz,Y., Meloan CE. 1994. *Food Analysis*: Theory and Practice, 3rd edition. Chapman & Hall, New York.

Porsch, B. 1993. Experimental Consequences of Differences between Composition of the Sample Solvent and of the Mobile-Phase in. *Journal of Liquid Chromatography*. 16 (16):3409-3421.

Prasad, K. N., L. Y. Chew, H. Khoo, B. Yang, A. Azlan, and A. Ismail. 2011. Carotenoids and Antioxidant Capacities from *Canarium odontophyllum miq*. Fruit. *Food Chemistry*. 124 (4):1549-1555.

Quackenbush, F. W., and R. L. Smallidge. 1986. Nonaqueous Reverse Phase Liquid-Chromatographic System for Separation and Quantitation of Provitamins-A. *Journal of the Association of Official Analytical Chemists*. 69 (5):767-772.

Rangel-Yagui, C. d. O., E. D. G. Danesi, J. C. M. de Carvalho, and S. Sato. 2004. Chlorophyll Production from *Spirulina platensis*: Cultivation with Urea Addition by Fed-Batch Process. *Bioresource Technology*. 92 (2):133-141.

Rezanka, T., J. Olsovska, M. Sobotka, and K. Sigler. 2009. The Use of APCI-MS with and Other Separation Techniques for Identification of Carotenoids and Related Compounds. *Current Analytical Chemistry*. 5 (1):1-25.

Richmond, A. 1990. Large Scale Microalgal Culture and Applications, pp. 269-330. In Round, M. and S. Chapman (Eds.). *Progress in Phycological Research*. Biopress, Bristol.

Rivera, S., F. Vilaró, and R. Canela. 2011. Determination of Carotenoids by Liquid Chromatography/Mass Spectrometry: Effect of Several Dopants. *Analytical and Bioanalytical Chemistry*. 400 (5):1339-1346.

Rodriguez-Amaya, D.B. 1999a. A Guide to Carotenoid Analysis in Foods. ILSI Press, Washington DC.

Sachindra, N. M., E. Sato, H. Maeda, M. Hosokawa, Y. Niwano, M. Kohno, and K. Miyashita. 2007. Radical Scavenging and Singlet Oxygen Quenching Activity of Marine Carotenoid Fucoxanthin and Its Metabolites. *Journal of Agricultural and Food Chemistry*. 55 (21):8516-8522.

Sánchez, J., J. Fernández, F. Acién, A. Rueda, J. Pérez-Parra, and E. Molina. 2008. Influence of Culture Conditions on the Productivity and Lutein Content of the New Strain *Scenedesmus almeriensis. Process Biochemistry*. 43 (4):398-405.

Sánchez-Luna, L. D., A. Converti, G. C. Tonini, S. Sato, and J. de Carvalho. 2004. Continuous and Pulse Feedings of Urea as a Nitrogen Source in Fed-Batch Cultivation of *Spirulina platensis*. *Aquacultural Engineering*. 31 (3):237-245.

Schiedt, K., S. Liaaen-Jensen (1995) Isolation and analysis.In Britton G, Liaaen-Jensen S, Pfander H. (eds), *Carotenoids*:Isolation and Analysis, vol 1A. Birkhäuser Verlag, Basel, pp 81-108.

Scott, K. J. 1992. Observations on Some of the Problems Associated with the Analysis of Carotenoids in Foods by. *Food Chemistry*. 45 (5):357-364.

Shi, X. M., F. Chen, J. P. Yuan, and H. Chen. 1997. Heterotrophic Production of Lutein by Selected *Chlorella* Strains. *Journal of Applied Phycology*. 9 (5):445-450.

Shi, X. M., H. J. Liu, X. W. Zhang, and F. Chen. 1999. Production of Biomass and Lutein by *Chlorella protothecoides* at Various Glucose Concentrations in Heterotrophic Cultures. *Process Biochemistry*. 34 (4):341-347.

Shi, X. M., X. W. Zhang, and F. Chen. 2000. Heterotrophic Production of Biomass and Lutein by *Chlorella protothecoides* on Various Nitrogen Sources. *Enzyme and Microbial Technology*. 27 (3-5):312-318.

Soletto, D., L. Binaghi, A. Lodi, J. Carvalho, and A. Converti. 2005. Batch and Fed-Batch Cultivations of *Spirulina platensis* using Ammonium Sulphate and Urea as Nitrogen Sources. *Aquaculture*. 243 (1):217-224.

Stengel, E., and C. Soeder. 1975. Control of Photosynthetic Production in Aquatic Ecosystems. Cambridge University Press. Cambridge. England.

Strati, I. F., and V. Oreopoulou. 2011. Effect of Extraction Parameters on the Carotenoid Recovery from Tomato Waste. *International Journal of Food Science and Technology*. 46 (1):23-29.

Su, Q., K. Rowley, C. Itsiopoulos, and K. O'dea. 2002. Identification and Quantitation of Major Carotenoids in Selected Components of the Mediterranean Diet: Green Leafy Vegetables, Figs and Olive Oil. *European Journal of Clinical Nutrition*. 56 (11):1149-1154.

Swanson, J.E. and Parker, R.S., Biological effects of carotenoids in humans, in Handbook of Antioxidants, Cadenas, E. and Packer, L. (Eds.), Marcel Dekker Inc., New York, Basel, Hong Kong, 1996, 337–367.

Takyi, E.E.K. Bioavailability of Carotenoids from Vegetables versus Supplements in Vegetables, Fruits and Herbs in Health Promotion, CRC Press, Boca Raton, FL.

Van Breemen, R. B., C. R. Huang, Y. Tan, L. C. Sander, and A. B. Schilling. 1996. Liquid Chromatography/Mass Spectrometry of Carotenoids Using Atmospheric Pressure Chemical Ionization. *Journal of Mass Spectrometry*. 31 (9):975-981.

Wang, C., S. Chang, B. S. Inbaraj, and B. Chen. 2010. Isolation of Carotenoids, Flavonoids and Polysaccharides from *Lycium barbarum L*. and Evaluation of Antioxidant Activity. *Food Chemistry*. 120 (1):184-192.

Wei, D., F. Chen, G. Chen, X. Zhang, L. Liu, and H. Zhang. 2008. Enhanced Production of Lutein in Heterotrophic *Chlorella protothecoides* by Oxidative Stress. *Science in China Series C: Life Sciences*. 51 (12):1088-1093.

Weller, P., and D. E. Breithaupt. 2003. Identification and Quantification of Zeaxanthin Esters in Plants Using Liquid Chromatography-Mass Spectrometry. *Journal of Agricultural and Food Chemistry*. 51 (24):7044-7049.

Yan, X., Y. Chuda, M. Suzuki, and T. Nagata. 1999. Fucoxanthin as the Major Antioxidant in *Hijikia fusiformis*, a Common Edible Seaweed. *Bioscience, Biotechnology, and Biochemistry*. 63 (3):605-607.

Zailanie, K., and H. Purnomo. 2011. Fucoxanthin Content of Five Species Brown Seaweed from Talango District, Madura Island. *Journal of Agricultural Science and Technology A*: 1103.

Ayşegül ERDOĞAN

Date and Place of Birth: 15.09.1980, İzmir
Nationality: Turkish (TC)
E-mail: aysegul_erdogan@live.com

EDUCATION

[2007-2013] **Ph.D.** (Faculty of Science, Department of Chemistry)
İzmir Institute of Technology
Dissertation: Investigation of Carotenoid Contents of Various Microalgae by Chromatographic/Spectroscopic Methods

[2004-2007] **M.Sc.** (Faculty of Science, Department of Chemistry)
İzmir Institute of Technology
Dissertation: Preconcentration of Heavy Metals in Environmental Samples by Biosorption and Determination by Atomic Spectrometry

[1999-2004] **B.Sc. (**Faculty of Science, Department of Chemistry)
İzmir Institute of Technology

ACADEMIC POSITIONS HELD

[2005-2013] **Research Assistant**
İzmir Institute of Technology
Faculty of Science, Department of Chemistry, İzmir, Turkey
Assisted undergraduate courses: General Chemistry, General Chemistry Laboratory I&II, Analytical Chemistry Laboratory I&II, Instrumental Analysis Laboratory, Inorganic Chemistry Laboratory.

PUBLICATIONS

Şeker A., Shahwan T., Eroğlu A. E., Yılmaz S., Demirel Z., Conk Dalay M. (2008). "Equilibrium, thermodynamic and kinetic studies for the biosorption of aqueous lead (II), cadmium(II) and nickel(II) ions on *Spirulina platensis*" *J. Haz. Mat., 154:*973-980.