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Progress in Carotenoid Research

Edited by Leila Queiroz Zepka, Eduardo Jacob-Lopes and Veridiana Vera De Rosso

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http://dx.doi.org/10.5772/intechopen.73775
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Contributors

Amy Lo, Denys Kurinnyi, Stanislav Rushkovsky, Olena Demchenko, Mariya Pilinska, Congqiang Zhang, Antonio Pérez-Gálvez, María Roca, Wenjiang Huang, Xianfeng Zhou, Weiping Kong, Huichun Ye, Adela Mora-Gutierrez, Rahnat Attaie, Maryuri Nuñez De González, Lucia Maria Jaeger De Carvalho, Masaki Honda, Hayato Maeda, Tetsuya Fukaya, Motonobu Goto, Jennifer Alcaino, Pamela Cordova, Marcelo Baeza, Victor Cifuentes, Andrêssa Fernandes, Leila Queiroz Zepka, Tatiele Casagrande Do Nascimento, Eduardo Jacob-Lopes, Veridiana Vera De Rosso, Zhiwei Men, Nan Gong, Zuowei Li, Chenglin Sun

Notice

First published in London, United Kingdom, 2018 by IntechOpen
eBook (PDF) Published by IntechOpen, 2019
IntechOpen is the global imprint of INTECHOPEN LIMITED, registered in England and Wales, registration number: 11086078, The Shard, 25th floor, 32 London Bridge Street
London, SE19SG – United Kingdom
Printed in Croatia

British Library Cataloguing-in-Publication Data
A catalogue record for this book is available from the British Library

Additional hard and PDF copies can be obtained from orders@intechopen.com

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p. cm.

Print ISBN 978-1-78923-716-0

Online ISBN 978-1-78923-717-7

eBook (PDF) ISBN 978-1-83881-752-7

Meet the editors

Prof. Leila Queiroz Zepka is currently an associate professor at the Department of Food Technology and Science, Federal University of Santa Maria. She received her master degree in Food Engineering from the Federal University of Rio Grande and doctoral degree in Food Science from the State University of Campinas. She has more than 15 years of teaching and research experience. She is a technical and scientific consultant of several companies, agencies, and scientific journals. She has published 340 publications and communications, which includes 8 books, 25 book chapters, 60 original research papers, and 250 research communications in international and national conferences and registered 10 patents. Her research interest includes microalgal biotechnology with an emphasis on biomolecules and natural pigments.

Prof. Veridiana Vera de Rosso is currently an associate professor at the Department of Biosciences, Federal University of São Paulo. She received her master degree in Food Engineering from the Federal University of Rio Grande, doctoral degree in Food Science from the State University of Campinas, and postdoctoral degree from the State University of Campinas. She has more than 15 years of teaching and research experience. She is a technical and scientific consultant of several agencies and scientific journals. She has published 150 publications and communications, which includes 1 book, 2 book chapters, 55 original research papers, and 70 research communications in international and national conferences and registered 1 patent. Her research interest includes food chemistry with an emphasis on natural pigments.

Prof. Eduardo Jacob-Lopes is currently an associate professor at the Department of Food Technology and Science, Federal University of Santa Maria. He received his master degree in Food Engineering from the Federal University of Rio Grande, doctoral degree in Chemical Engineering from the State University of Campinas, and postdoctoral degree from the State University of Campinas. He has more than 15 years of teaching and research experience. He is a technical and scientific consultant of several companies, agencies, and scientific journals. He has published 450 publications and communications, which includes 8 books, 28 book chapters, 75 original research papers, and 350 research communications in international and national conferences and registered 12 patents. His research interests include biotechnology and bioengineering with an emphasis on microalgal biotechnology.

Contents

Preface

Currently, there is information on 1182 natural carotenoids scattered with 697 bioresources. The original chemical fingerprints are currently investigated, making it easy to classify chemical modification patterns in carotenoid structures, to search for similarities, and to predict some of the biological functions of carotenoids such as photosynthetic pigment, photoprotective activity, provitamin A, antioxidant, spectral filter of the eye retina, anti-photosensitizing agent, membrane stabilizer, anticarcinogenic activity, induce cell differentiation, antimicrobial activity, anti-diabetes activity, anti-obesity, anti-inflammatory, immune response enhancement, feeding deterrent activity, allelochemicals, retinoic acid receptors, and transcription activation besides sensory functions related to odor, taste, and color. Our goal in this book is to amplify the understanding of how organisms are related to carotenoids either through aspects related to basic science or applied technology to this research field.

Leila Queiroz Zepka
Federal University of Santa Maria
Santa Maria, Brazil

Veridiana Vera de Rosso
Federal University of São Paulo
Santos, Brazil

Eduardo Jacob-Lopes
Federal University of Santa Maria
Santa Maria, Brazil

Chapter 1

Carotenoids: A Brief Overview on Its Structure, Biosynthesis, Synthesis, and Applications

Andrêssa Silva Fernandes,
Tatiele Casagrande do Nascimento,
Eduardo Jacob-Lopes, Veridiana Vera De Rosso and
Leila Queiroz Zepka

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.79542

Abstract

Currently, 1178 natural carotenoids have been properly characterized and reported in the literature, which present a huge structural diversity and physicochemical properties. This number comprises a wide distribution of these biomolecules in approximately 700 source organisms including plants, bacteria, fungi, and algae. Besides having a wide applicability as natural dyes, some carotenoids such as β-carotene already have another well-established application such as provitamin A activity. However, due to the structural diversity of these molecules, there are still numerous biochemical and physiological functions to be associated with this class of compounds. Accordingly, these characteristics enable a wide applicability, what drives the global carotenoid market. Thus, with the primary objective of addressing aspects regarding to basic science and applied carotenoid technology, a comprehensive description of the biology, biochemistry, and chemistry of these compounds will be described in this chapter.

Keywords: carotenoid, structure, synthesis, biosynthesis, industrial application

1. Historical aspects of carotenoids

It is often stated that without carotenoids, life in an oxygenic atmosphere would not be possible, and we would not exist. Thereby, over millions of years, the living organism chloroplasts maintained collections of carotenoids to protect the intricate and delicate photosynthetic apparatus against destruction by photooxidation [1, 2].

According to Britton et al. [1], it can be considered that the study of carotenoids exceeds 200 years of history. Was Braconnot in 1817 carried out the first investigation in paprika? The following year, Aschoff isolated from the saffron, the "crocin," apocarotenoid which we now know as bixin. In 1823, Goebel's research on crab (*Brachyura*) suggested for the first time the presence of these isoprenoids in animals. Later, after investigations with carrots (*Daucus carota* L.), from which the term carotenoids derives, Wackenroder in 1831 isolated and described for the first time carotene with structure C40, now β-carotene. Shortly thereafter in 1837, Berzelius introduced the term xanthophyll due to its presence in autumn leaves. After, Kraus and Millardet in 1843 made the first investigation of carotenoids into cyanobacteria. Only 30 years later, lycopene was isolated for the first time from fruits of *Tamus communis* by Hartsen.

However, it was in the early twentieth century that a milestone occurred in the history of carotenoids. The Russian botanist Tswett in 1906 took the first step in the chromatographic technique of separation of these pigments, which together with the introduction of mass spectrometry (MS) in 1965 and high-performance liquid chromatography (HPLC) in 1971 provided a great advance in research [1, 3]. From this and with the advent of chromatographic methods and refinements in spectroscopy, the isolation and identification of carotenoids expanded greatly.

According to the last compilation, approximately 1178 naturally occurring carotenoids have been reported with 700 source organisms [4].

2. Structure, biosynthesis, synthesis, and application

In fact, it is well known that carotenoids are naturally synthesized by all photosynthetic organisms and nonphotosynthetic some, like bacteria, archaea, and fungi, which exhibit a complex carotenogenic metabolism [5].

They are classified according to the number of carbons that constitute their structure into carotenoids C30, C40, C45 and C50, but only the C40 carotenoids are those found in nature more abundantly and consequently, are those more approached in the literature. Moreover, C40 carotenoids are biosynthesized by organism eukaryotes, archaea, and bacteria, and their chemical structures are constituted by a diverse range of terminal groups [6].

Conversely, C30 and C50 carotenoids are biosynthesized by archaea and bacteria and only contain 6 and 10 C5 isoprenoid units, respectively. By contrast, only the bacteria are responsible for synthesizing C45 carotenoids composed of nine isoprenoid units [3, 6].

More than 100 naturally occurring apocarotenoids with diverse structural and functional properties have been reported. An apocarotenoid is a carotenoid in which the normal C40 structure has been shortened by the removal of fragments from one or both ends [7, 8]. Natural examples are bixin (C25 compound), the major pigment of the food colorant annatto, and crocetin (C20 compound), the main yellow coloring component of saffron [9, 10]. Lycopene, β-carotene, and zeaxanthin are the precursors of the main apocarotenoids described to date, which include bixin, crocetin, abscisic acid, strigolactone, and mycorradicin [10]. Vitamin A

is also considered an example of apocarotenoid, because it is the product of the symmetrical oxidative cleavage of β-carotene [7].

The formation of these carotenoid derivatives occurs via enzymatic and nonenzymatic oxidative cleavage of carotenoids [11, 12]. Carotenoid cleavage dioxygenases (CCDs) catalyze carotenoid cleavage at specific double bonds, typical act by incorporating oxygen atoms into adjacent carbon atoms along the conjugated carotenoid backbone. Some CCD cleavage reactions require isomerization to form substrate isomers favorable for cleavage [13]. On the other hand, nonenzymatic apocarotenoid formation can occur via singlet oxygen attack, primarily on β-carotene [14]. In addition, peroxidases and lipoxygenases are also reported to form apocarotenoids [15].

Regardless of metabolic origin, apocarotenoids present important biological functions, such as plant-environment interactions such as the attraction of pollinators and the defense against pathogens and herbivores. Also, include volatile aromatic compounds that act as repellents, chemoattractants, growth stimulators, and inhibitors, as well as the phytohormones abscisic acid and strigolactones [16]. Moreover, these isoprenoids are associated with other processes positively affecting human health were identified as responsible for inhibiting the lipid peroxidation and prevention of cancer and other degenerative diseases [14, 17, 18].

Nonapocarotenoid carotenoid cleavage products include norcarotenoids, which lack one, two or three carbon atoms in the central hydrocarbons skeleton (C40) [3]. The primary determinant is the number of carbon atoms formally lost from the C40 carotenoid skeleton [5]. An example is the peridinin, is one of the most complex carotenoids, a C37-norcarotenoid possessing (Z)-γ-ylidenebutenolide and allene functions. In addition, it has five chiral centers, including an epoxide ring [19].

Another subclass is that of secocarotenoids, in which a bond between two adjacent carbon atoms except between C(I) and C(6) in a ring has been broken [3, 5]. The semi-β-carotenone ($C_{40}H_{56}O_6$) is an example identified as the product of β-carotene ($C_{40}H_{56}$) oxidation in permanganate solutions [20].

In addition, isoprenoid structures with more than 40 carbon atoms are also reported. The rare C50 carotenoids are synthesized by the addition of two dimethylallyl pyrophosphate (DMAPP) molecules to C(2) and C(2′) of the respective C40 carotenoid [21]. These compounds have been mainly isolated from *Halobacteria, Halococcus*, and *Pseudomonas strain* and *Actinomycetales* [22]. The first C50 carotenoid discovered, decaprenoxanthin, was isolated from *Flavobacterium dehydrogenans* [23].

As shown in **Figure 1**, structurally, carotenoids have different terminal groups, of which there are seven: ψ, β, γ, ε, ϕ, χ, and κ, which may constitute the ends of the principal polyene chain of the structure of these molecules. In general terms, the terminal rings β, γ, and ε rings are formed from ψ ends, whereas ϕ, χ, and κ rings are formed from β end groups [6, 24].

Lycopene is the common precursor structure for the synthesis of cyclic and bicyclic carotenoids. Cyclization of this molecule is a branching point in carotenoid biosynthesis, where β-, γ- and ε-end groups are formed by proton loss from alternative positions in the

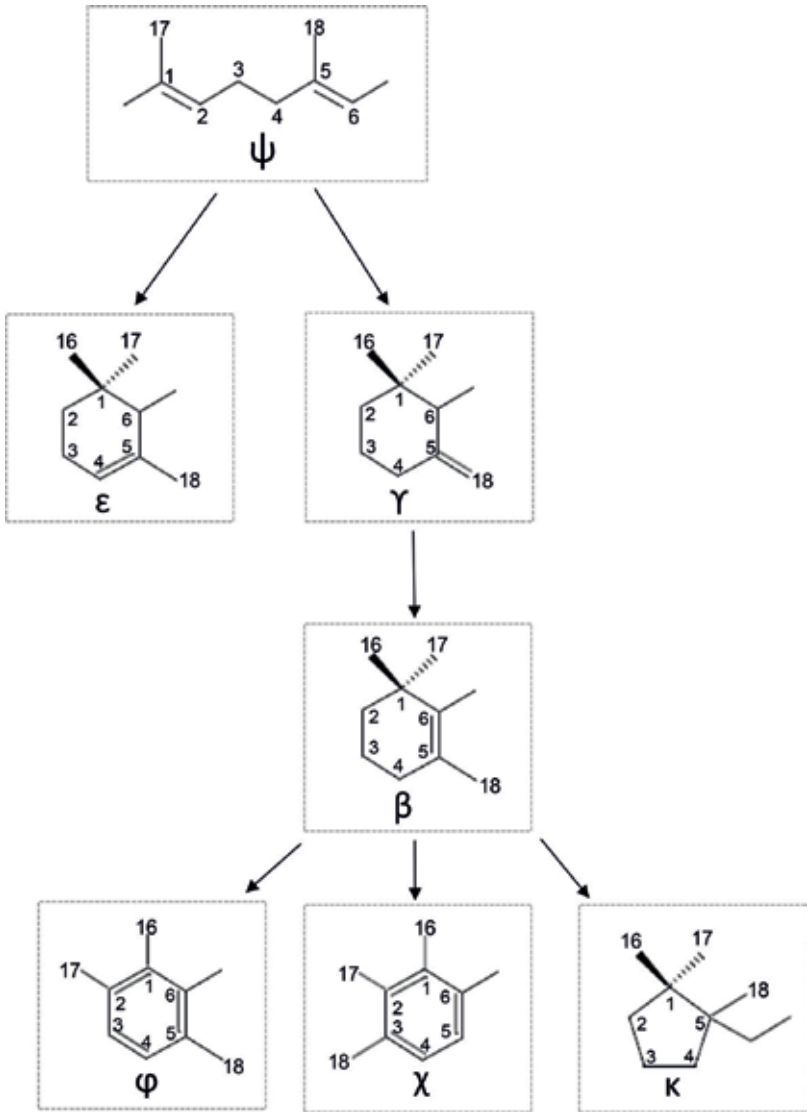
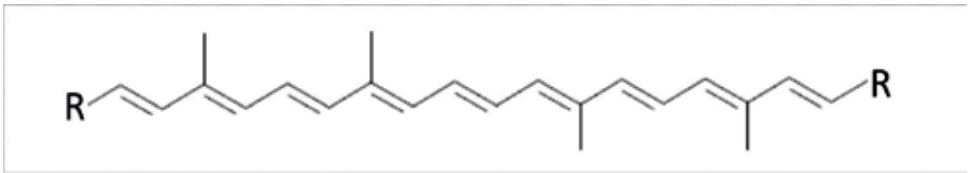


Figure 1. Different terminal rings of carotenoids.

same transient carbon ion intermediate. This cyclization process is catalyzed by the action of the enzymes lycopene cyclases [6]. The most prominent carotenoids with the ψ-, β-, γ- and ε-ends groups are licopene, β-carotene, γ,γ-carotene, and ε,ε-carotene, respectively (**Figure 2**).

The biosynthetic process of rings ϕ and χ (from ring β) occurs by the migration of methyl groups from C1 to C2 and dehydrogenation. In addition, migration of the methyl group from C5 to C3 occurs in the ring χ. Isorenieratene and renierapurpurin are representative carotenoids with the ϕ and χ end groups [24].

In addition, carotenoids with κ terminal group are biosynthesized from 3-hydroxy-5,6-epoxy-β rings found in violaxanthin and antheraxanthin. This terminal group is characteristic of capsorubin, capsanthin, and cryptocapsin, isolated from paprika (*Capsicum annuum*) [24].



Type	End group (R)	Carotenoid
Acyclic	ψ	Lycopene
Cyclohexene	β	β-Carotene
Cyclohexene	ε	ε,ε-Carotene
Methylenecyclohexane	γ	γ,γ- Carotene
Cyclopentane	κ	Capsorubin
Aryl	χ	Renierapurpurin
Aryl	ϕ	Isorenieratene

Figure 2. Carotenoids with different terminal groups.

However, even after decades of studies with carotenoids, the studies that report on the enzymatic and chemical mechanisms responsible for the constitution of the terminal moieties of these molecules are limited, despite its relevance to the full meaning of a biosynthesis of carotenoids [24].

Considering the chemical elements present in the structure of carotenoids, they can be classified into carotenes and xanthophylls as shown in **Figure 3**. The carotenes are compounds which contain only hydrocarbons in its structure (e.g., β-carotene and lycopene). On the other hand, the xanthophylls are oxygenated carotenoids, which contain different functional groups such as an epoxy (violaxanthin, neoxanthin, and fucoxanthin), hydroxy (lutein and zeaxanthin), keto (astaxanthin and canthaxanthin), and methoxy (spirilloxanthin) functional groups. In turn, xanthophylls are among the main carotenoids in photosynthetic tissues [3, 25].

According to these modifications, these functional groups containing oxygen affect the biological functions and the solubility of carotenoids, making xanthophylls more polar than carotenes, thus allowing their separation using many types of chromatographs [7].

The formation of functional groups of the xanthophylls occurs naturally by an enzymatic reaction. The hydroxy group formation occurs through ring-specific hydroxylation reactions and is normally catalyzed by carotene β-hydroxylase enzymes of the nonheme di-iron (BCH) type. In the case of β-carotene, two sequential hydroxylations of the β rings produce first β-cryptoxanthin and then zeaxanthin. The same enzymes can also participate in the synthesis of lutein [26, 27].

Zeaxanthin epoxidase (ZEP) introduces epoxy groups in the rings of zeaxanthin, resulting in the formation of violaxanthin, which undergoes the introduction of a double allenic bond in the molecule producing neoxanthin in one step catalyzed by neoxanthin synthase (NSY) [3, 26].

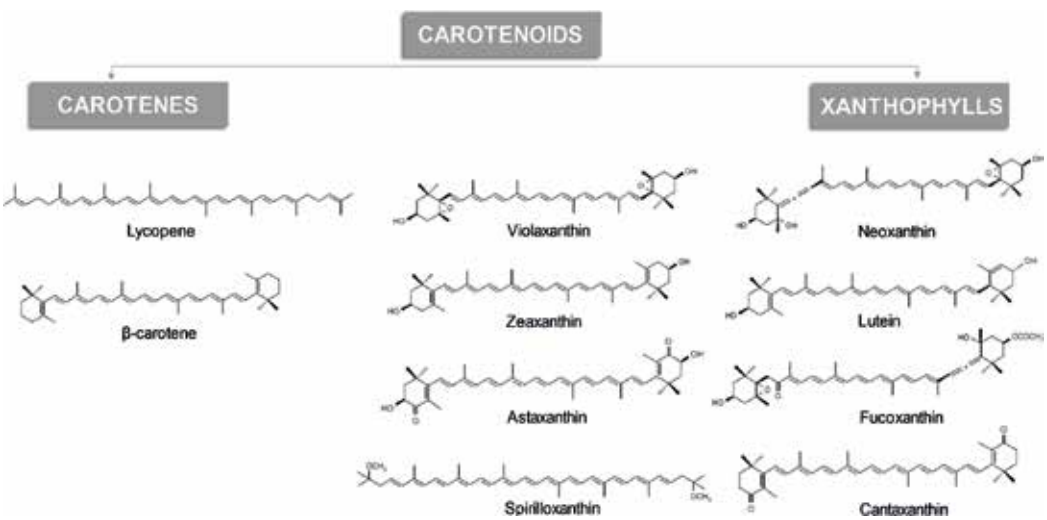


Figure 3. Examples of carotenes and xanthophylls.

Epoxy carotenoids comprise a large group of xanthophylls and are widely encountered in foods [28].

On the other hand, there are the ketocarotenoids that are produced by some algae and cyanobacteria, and are rare in plants [29]. These compounds have the ketone group inserted into the molecule by the enzyme beta-caroteneketolase. Ketocarotenoids, echinenone, and astaxanthin are the examples. Referring to astaxanthin, it still suffers action from beta-carotene hydrolase, since its structure is composed of two hydroxyls [30]. Ketocarotenoids are strong antioxidants that are chemically synthesized and used as dietary supplements and pigments in the aquaculture and nutraceutical industry [31].

Methoxy-carotenoids are previously synthesized from bacteria via enzymatic methoxylation [32], and the spirilloxanthin is an example [33]. Though the 3-methoxy-zeaxanthin has been reported in the human macula, your metabolic origins are unknown. It is suggested that methoxy xanthophyll originates from they do make metabolic changes to carotenoids acquired of the diets [32].

Besides with all the distinct conformations of carotenoids described above, these pigments may be associated to other molecules, including fatty acids (carotenoid esters), sugars (glycosylated carotenoids) or even proteins (carotenoproteins).

Carotenoids are naturally found in both free forms and esterified with fatty acids in many fruits, flowers, animals, microorganisms, and algae. For an ester to be formed, the carotenoid must have at least one hydroxyl group, since the ester linkage is formed when a carboxylic acid (fatty acid) reacts with an alcohol group (hydroxylated xanthophyll), with the elimination of a water molecule [34]. This process increases the lipophilicity of the molecule. During carotenoid biosynthesis to suggest that the xanthophyll esterification with fatty acids, it is most likely to be catalyzed by esterases or xanthophyll acyl transferases [35].

In many fruits, some plant organs, and tubers, the xanthophylls are typically found esterified with fatty acids [34, 36]. Similarly, there are carotenoids associated with sugar moieties, as it is the case of crocetin. On the other hand, some carotenoids can form complexes with proteins (carotenoproteins) that are water soluble and appear to stabilize carotenoids, as occurs with some crustaceans (astaxanthin-crustacyanine complex) [37].

Still referring to the patterns of chemical modifications, there are allene carotenoid and acetylene carotenoid structures (see **Figure 4**). In some important examples of naturally occurring carotenoids, the polyene chain is modified by the presence of one or two acetylenic (—C≡C—) or allenic (—C≡C—) groups, what is common in many marine organisms. The marine, allenic carotenoid peridinin from phytoplankton and fucoxanthin from macroalgae and phytoplankton are the carotenoids produced in largest quantity in nature [38]. Acetylenic carotenoids are synthesized de novo only in microalgae; crocoxanthin and diatoxanthin are examples of these structures [39, 40].

Given the presence of double bonds in carotenoid molecules, multiple geometrical (*cis*/*trans* or *Z*/*E*) isomers could be formed, which differ considerably in their chemical shape. However, most carotenoids found in nature are primarily in the more stable all-*trans* configurations; a small proportion of *cis* isomers is encountered [5, 12].

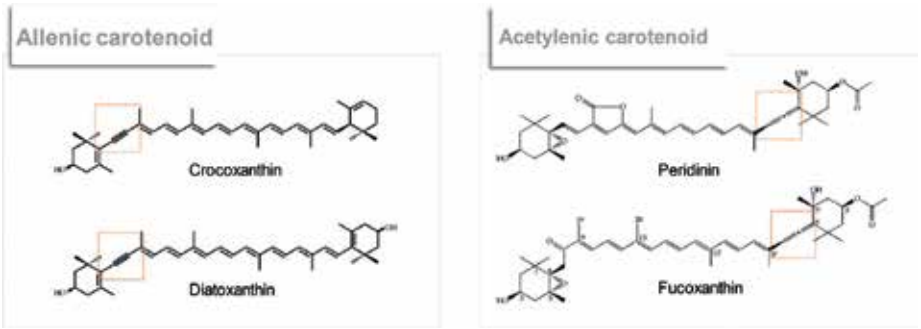


Figure 4. Carotenoids with acetylenic and allenic structure.

Theoretically, each carbon-carbon double bond in the polyene chain of carotenoids may exhibit E-Z isomerization. However, some double links like C-7,8, C-11,12, C-7′,8′, and C-11′,12′ are prevented from undergoing isomerization Z due to a steric hindrance between a hydrogen atom and a methyl group [28]. Thus, the Z-isomers of symmetrical β-carotene and zeaxanthin commonly found are the 9-*Z*-, 13-*Z*-, and the 15-*Z*-isomers, the formation of which has relatively little hindrance as it comes from two hydrogen atoms [28].

However, the C-5,6 double bond in the acyclic lycopene is unhindered, and 5-*Z*-lycopene is increasingly detected, along with the 9-*Z*-, 13-*Z*-, and the 15-*Z*-isomers [41].

Although the presence of *cis* isomers is recognized due to the isomerization caused by heat or light sources, there are some carotenoids that can occur naturally. Interestingly, they have different biological potency than their trans counterparts (e.g., lower pro vitamin A activity) [3]. Phytoene and phytofluene, which have the 15-*Z* configuration in most natural sources, are examples of carotenoids less thermodynamically stable [42]. Another example is bixin (see **Figure 5**), an apocarotenoid which occurs naturally in the *Z* form [43].

By contrast, the presence of one or more centers or axes of chirality in their molecules, some carotenoids can undergo geometric isomerization, which evidence the formation of optical (*R*/*S*) isomers. Zeaxanthin and astaxanthin are typical examples of carotenoids, in which this isomerization may occur.

Two optical isomers (3*R*-3′*R*)-zeaxanthin and (3*R*-3′*S*)-zeaxanthin, commonly referred to as *meso*-zeaxanthin and (3*S*, 3′*S*)-zeaxanthin are found in the macula lutea of the human retina [5]. Conversely, optical isomers different from astaxanthin, 3*S*, 3′*S*, 3*R*, 3′*S* (*meso*), and 3*R*, 3′*R*, in varying proportions are found in marine organisms (see **Figure 6**) [5].

At the biosynthetic level, more than 95% of all known carotenoids are formed using the same C5 building block, the isoprene (C5H8) unit, from which isopentenyl pyrophosphate (IPP) and its allylic isomer dimethylallyl pyrophosphate (DMAPP) are produced. Thus, in the route of the synthesis of isoprenoids, three molecules of IPP are sequentially added to DMAPP by prenyl transferase enzymes to yield geranylgeranyl-pyrophosphate (GGPP, C20). From this stage, the specific carotenoid biosynthetic pathway starts with condensation occurs of two molecules of GGPP, by phytoene synthase (PSY) to produce the first colorless carotenoid

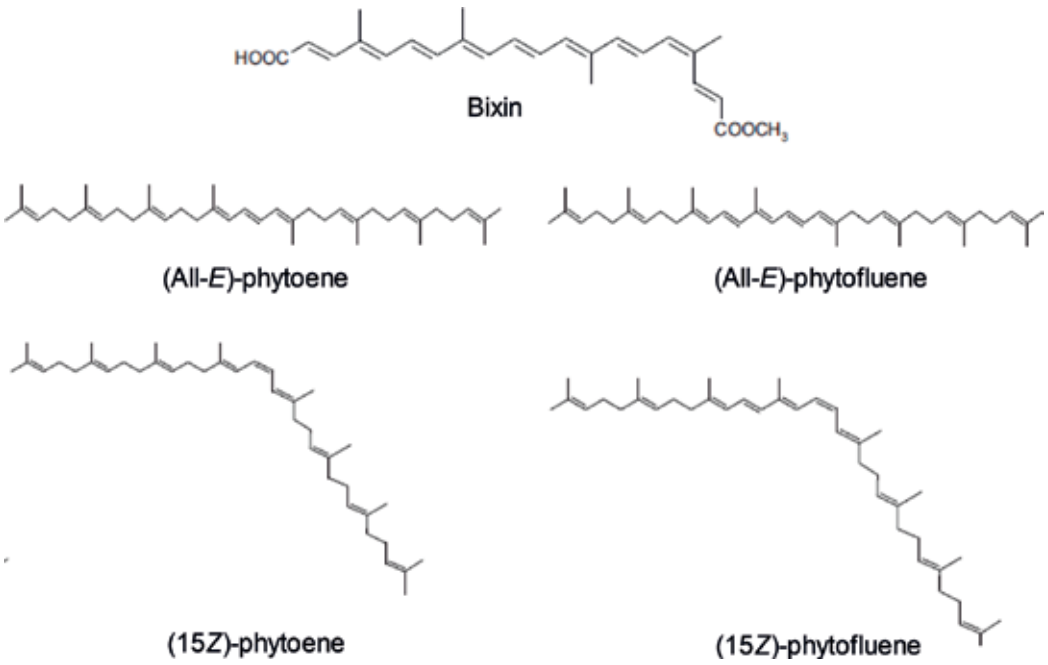


Figure 5. Geometrical isomers.

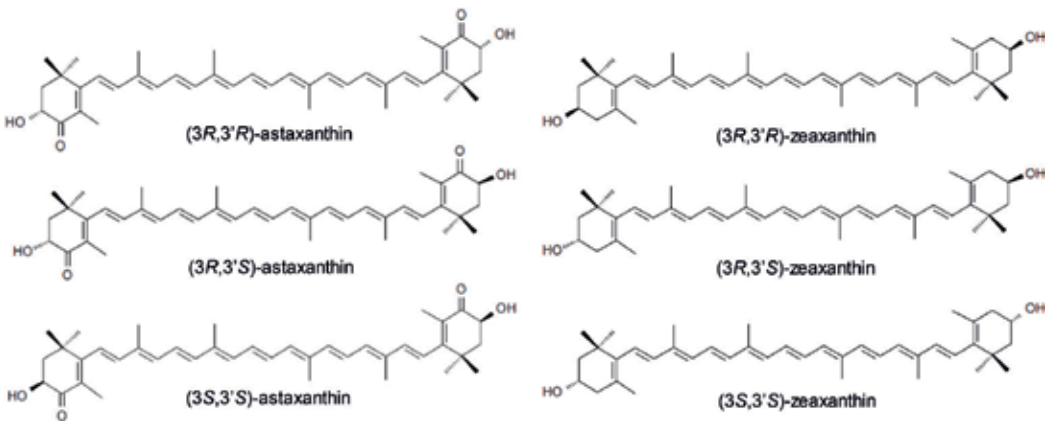


Figure 6. Optical isomers.

15-*cis*-phytoene (C40). These precursors are produced by two independent pathways in photosynthetic beings: the mevalonate (MVA) pathway and the methylerythritol 4-phosphate (MEP) pathway [3].

In contrast, approximately 5% of the biosynthesis of the other carotenoids (C30) occurs from farnesyl pyrophosphate (FPP), an intermediate precursor of geranylgeranyl-pyrophosphate (GGPP) or by the oxidative cleavage process of C40 carotenoids [21, 44].

Additionally, the carotenoids biosynthesized by different organisms are derived through a series of chemical and enzymatic modifications from the phytoene, such as reactions of desaturations, cyclizations, hydroxylations, glycosylation, oxidization, dehydrogenation, migration of double bonds, rearrangement, and epoxidations, as exemplified above [45]. These modifications are catalyzed by a number of enzymes which fall into few classes based on the type of transformation they catalyze such as geranylgeranyl pyrophosphate synthase, phytoene synthase, carotene desaturase, and lycopene cyclase. Modification of carotenes is further catalyzed by β-carotene ketolase and β-carotene hydrolase to generate various C40 carotenoids. Thus, all of these modifications contribute to yield a family of more than 1178 compounds widely distributed in nature [4, 46].

Of the total number of naturally occurring carotenoids, only eight are produced synthetically at industrial level. Between them C40 carotenoids: lycopene, β, β-carotene, (3R,3′R)-zeaxanthin, canthaxanthin, and astaxanthin; and three apocarotenoids: ß-apo-8′-carotenal, ethyl ß-apo-8′-carotenoate, and citranaxanthin [47]. For the chemical synthesis, several building concepts are possible. However, on industrial scale, only few of them have been applied successfully. The reactions of Grignard elaborated by Hoffman-La Roche in 1954 and reactions of Wittig developed by Badische Anilin- & Soda-Fabrik (BASF) in 1960, were the main reactions of syntheses employed on an industrial scale; however, Wittig reaction dominates the market currently [24, 48].

All chemically synthesized C40 carotenoids have symmetric structures, and this is explained by the fact that all structures have identical end groups at their ends. Due to these characteristics, they are efficiently produced by double Wittig condensation of a symmetrical C10-dialdehyde as the central C10-building block with two equivalents of an appropriate C15-phosphonium salt. In addition to these synthetic steps, these mixtures of isomers are thermally isomerized, in heptane or ethanol, for the full formation of all-trans/E configurations, since during the process, certain amounts of cis/Z stereoisomers are formed [24, 48]. Additionally, to use Grignard compounds, it is necessary to combine one diketone molecule and two methanol molecules, thereafter compound containing 40 carbon atoms is obtained [49].

Other methods of the synthesis of carotenoids include the hydroxylation of canthaxanthin, a C10 + C20 + C10 synthesis via dienolether condensation, and the isomerization of a lutein extracted from marigold to zeaxanthin and then oxidation to astaxanthin [48].

Apart from β, β-carotene, the other synthetically produced carotenoids are manufactured mostly by the companies Hoffmann-La Roche and BASF [47].

Furthermore, to synthetically traded carotenoids, a portion of these pigments are obtained from natural sources such as lutein (marigold flowers), β-carotene (*Dunaliella salina*), astaxanthin (*Haematococcus* spp.), and Capsorubin (*Capsicum annuum*) (see **Table** 1) [1, 50]. β-carotene followed by lutein and astaxanthin lead the carotenoid market, which is projected to reach USD 1.53 Billion until 2021 [51].

In more recent times, the major commercial use of carotenoids has been as food and feed additives for coloration. They have also found some use in cosmetics and pharmaceutical products, but the most rapidly growing market now is health supplements, which in turn, provides a stimulus growing from production [1].

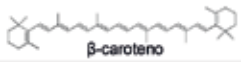
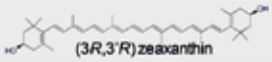
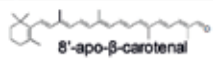
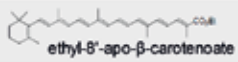
Carotenoid	Applications	Production Technology
 Lycopene	food coloration, nutritional supplement	chemical synthesis, biotechnology (Blakeslea trispora), isolation from tomato
 β-caroteno	food coloration (margarine, juice), nutritional supplement, feed additive (fertility, cattle)	chemical synthesis, biotechnology (*Dunaliella salina*)
 (3*R*,3′*R*) zeaxanthin	nutritional supplement (eye health)	chemical synthesis, isolation from natural sources
 Cantaxanthin	poultry (egg yolk and broiler pigmentation), aquaculture	chemical synthesis
 Astaxanthin	aquaculture (salmon pigmentation), dietary supplement, food coloration	chemical synthesis, biotechnology (*Haematococcus pluvialis*)
 8′-apo-β-carotenal	food coloration (cheese, dressings)	chemical synthesis
 ethyl-8′-apo-β-carotenoate	feed additive (egg yolk and broiler pigmentation)	chemical synthesis
 Citranaxanthin	feed additive (egg yolk and broiler pigmentation)	chemical synthesis

Table 1. Carotenoids industrial applications.

Author details

Andrêssa Silva Fernandes[1], Tatiele Casagrande do Nascimento[1], Eduardo Jacob-Lopes[1], Veridiana Vera De Rosso[2] and Leila Queiroz Zepka[1]*

*Address all correspondence to: zepkaleila@yahoo.com.br

1 Food Science and Technology Department, Federal University of Santa Maria, UFSM, Santa Maria, RS, Brazil

2 Department of Biosciences, Federal University of São Paulo, UNIFESP, Santos, SP, Brazil

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Chapter 2

Recent Developments in the Analysis of Carotenoids by Mass Spectrometry

Antonio Pérez-Gálvez and María Roca

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.79755

Abstract

Mass spectrometry has become an indispensable tool for the analysis of carotenoids in photosynthetic tissues, foods, and biological materials from different sources to accurately establish their pigment profile, to provide evidences to distinguish the different structural arrangements, and to obtain biological meaning from metabolic processes where carotenoids participate during the development of their natural functions and activities. The recent progresses in the hyphenated HPLC systems with hybrid mass spectrometers, which enhance the acquisition of independent and complementary physicochemical properties for the identification of carotenoids, are detailed in this chapter. A reasonable guide for the implementation of post-processing routines, assisted by modern software tools, and the key issues for the analysis of the characteristic product ions are also defined in this contribution to help the readers in the understanding of the potential capabilities of mass spectrometry in the field of carotenoid pigments.

Keywords: extraction methods of carotenoids, liquid chromatography, ionization procedures, mass spectrometry, metabolomics, fragmentation pathways and carotenoid identification, data analysis from mass spectra, software tools

1. Introduction

The research interest in the characterization of the carotenoid content does not exclusively aim to determine the identity or taxa of the species, or to perform the screening of complex and intriguing carotenoid structures that are consequence of the variable gene expression, but also to find and/or select those species that may fulfill the claim in an applicative field. The analytical technique(s) applied to unequivocally identify the individual carotenoids must

provide solutions to face several challenges, because the carotenoid profile usually contains a complex mixture of pigments, with a wide polarity range where the presence of isomeric forms or structurally related compounds like xanthophyll esters is frequent, as well as interfering compounds (chlorophylls and triacylglycerides) in the analysis of the sample. In some biological samples, as serum or animal tissues, the carotenoid concentration could be very low, even below the nanomolar concentration [1]. In addition, carotenoids are unstable in the presence of light, oxygen, or heat [2]. Such lability to high temperatures has limited the application of gas chromatography mass spectrometry (GC-MS) in the carotenoid analysis, in combination with the complexity of the derivatization reactions. However, GC-MS has been applied to report the presence of different low-molecular weight compounds from the metabolism of several carotenoids in biological studies [3–6] with interesting results.

Historically, carotenoids have been analyzed by thin layer chromatography (TLC), a simple and fast technique to separate carotenoids, which allowed their characterization during the end of the last century [7]. But the need of high concentration of samples, the open system feature, and the low resolution of this technique induced its replacement by analytical techniques with higher sensitivity and superior selectivity. Consequently, high-performance liquid chromatography (HPLC) coupled to photodiode-array or UV-visible detectors is the common analytical technique currently applied to achieve the separation of carotenoids and their subsequent identification (in most cases) and quantification considering the features of the absorption spectrum, the location of the main absorption bands and its shape or fine structure, and the chromatographic behavior. The UV-visible spectrum of carotenoids provides some structural information, but the complete characterization is not feasible exclusively in the basis of the spectroscopic properties. Many carotenoids share the same spectrum and when the structures are closely related, even the chromatographic behavior is similar. Hence, the hyphenation of HPLC with mass spectrometry (MS) has become the ideal analytical technique because MS allows the acquisition of the elemental composition and structural arrangement of the functional groups in each individual carotenoid, overcomes the problems of resolution and sensitivity when the amount of sample/compound is low, and reduces the effect of interfering components. A diverse collection of ionization strategies and configurations of the mass analyzer is feasible, and each one performs diverse intrinsic parameters (mass resolving power, mass accuracy, linear dynamic range and sensitivity) at different levels. While HPLC-MS has been successfully applied to the qualitative and quantitative analysis of carotenoids, and there is an abundant available information regarding the MS characteristics of carotenoids under different MS acquisition modes, the assignment of peaks from experimental data still requires an intensive manual labor. Conversely, the implementation of software tools to assist in the production of a positive list of identified carotenoids is still an occasional practice. In reference [8], the process to build a database with 22 standard pigments (carotenoids and chlorophylls) including their retention time and the theoretical *m*/*z* values of both the parent and most intense product ions is described. The experimental retention time and *m*/*z* values acquired from the MS analysis of ethanol extracts of *Porphyridium purpureum* were compared with the values in the database, and when the mass errors were below 5 ppm, the presence of the target compound was confirmed. However, it would be encouraged to follow the rules for validating the identification, not only considering a single physicochemical feature (chromatographic behavior, UV-vis spectrum, *m*/*z*…) but on a minimum of two independent and orthogonal data relative

to an authentic compound (standard) analyzed under identical experimental conditions [9] to match with the compounds analyzed in the sample (see Section 3.2). Indeed, complementary confidence is provided when more than one pair of orthogonal data are selected to perform the identification. In the case of HPLC-MS analysis, different pairs of independent and complementary physicochemical properties are available (retention time and mass spectrum, accurate mass and tandem MS, and accurate mass and isotopic pattern). With the available information regarding the MS characteristics of carotenoids, it is possible to automatically obtain a report of carotenoids from an in-house mass database created *ex profeso* that matches the experimental *m*/*z* values with the selected orthogonal characteristics of the former. These MS criteria are complemented with additional matching data (retention time and UV-visible spectrum) to ensure the identification. Indeed, it is possible to perform a screening for unknown carotenoids or those not included in the database by the molecular feature extraction algorithms and subsequently apply the same pairs of orthogonal data to the new *m*/*z* value(s).

Alternatively, analysis of carotenoids by direct infusion without the necessity of a previous chromatographic separation step has been also described. It is possible to distinguish the *cis-trans* isomers of lycopene and β-carotene by their fragmentation patterns with the use of electrospray ion mobility mass spectrometry, for example see [10]. In a similar fashion, reference [11] describes the isolation of zeaxanthin and its glucoside fatty acid esters (thermozeaxanthins and thermo-*bis*-zeaxanthins) to characterize mutants in the genes, which encode the enzymes performing the final carotenoid glycosylation and acylation steps. Nevertheless, the flow infusion mass spectrometry has some limitations [12]. In this sense, the combination of a multidimensional analytical approach based on UPLC-UV-ion mobility-MS proposed for the screening of natural pigments could be the last novelty [13]. The authors described this novel approach with the aim to increase the selectivity and specificity in the analysis, and to improve the characterization of carotenoids and chlorophylls in complex biological matrices.

2. Extraction of carotenoids and sample preparation for analysis

It is challenging to define a general and common method for carotenoid extraction. The optimal extraction depends not only on the polarity of the carotenoids, but also on the characteristics of the matrix (moisture content, presence of cell wall, etc.). Additional parameters to take into consideration are the environmental and health hazards issues (with some solvents), time consuming techniques, costs, or even feasibility. In any case, the analytical parameters that should be determined for the development of an analytical method are precision, accuracy, specificity, linearity, and limits of detection and quantification. The present section discusses in detail the advantages or disadvantages of each method and its applicability.

Liquid-liquid extraction or solvent extraction, and partitioning method is the more widespread technique applied for carotenoid extraction. The selection of the organic solvent nature is not a simple task. The polarity of the solvent and the characteristics of the matrix are determining parameters for the efficiency of the extraction. Typically, nonpolar solvents are used for nonpolar carotenoids (hexane for β-carotene extraction), while polar solvents are suitable for polar carotenoids (acetone for lutein extraction). However, the selection of the

proper solvent or mixture of solvent will subsequently require the optimization for each specific matrix. Thus, the hexane and the ethanol/hexane (4:3) are the solvents most frequently applied, although numerous alternatives have been assayed: acetone, ethanol, petroleum ether, tetrahydrofuran, diethyl ether, methanol, etc. The solvent or solvents mixture could be supplemented with antioxidants to avoid degradation and transformation of carotenoids during extraction. Butylated hydroxytoluene is the most well-known, although other antioxidants, such as pyrogallol and ascorbic acid, are also utilized.

Recent reviews have recapitulated the different extraction methods postulated for carotenoids [14–16]. One specific case is the esterified carotenoids [15]. Chemical hydrolysis cannot be applied if the aim is to determine the nature of fatty acids involved in the esterification, while the presence of triacylglycerides in the sample produces interferences in the subsequent chromatographic analysis. Therefore, the extraction methods mean the application of similar protocols applied for the extraction of free carotenoids, but the oily-dense nature of some matrices containing carotenoid esters requires clean-up steps. Different approaches have been set up, including the utilization of open column chromatography on silica gel [17] or magnesium oxide [18], enzymatic hydrolysis [19], solid-phase extraction [20], or even the use of semi-preparative isolation on C18 column and subsequent identification [21].

Although the most common method for the isolation of the carotenoid profile is solvent extraction, during the last decade, new techniques have raised becoming popular because of their *green* features and environmentally friendly practices. They include the microwaved-assisted extraction (MAE), ultrasound-assisted extraction (UAE), pressurized liquid extraction (PLE), pulse electric field-assisted extraction (PEF), supercritical fluid extraction (SFE), and enzyme-assisted extraction (EAE). The development of these techniques is mainly motivated by the specific difficulties for the extraction of lipophilic content from microalgae and seaweeds species. The cell walls and the extracellular material with different polysaccharide distributions, such as agars, carrageenans, fucoidans, and alginates, limit the mass transfer of carotenoids during conventional extraction processes. The biological characteristics of those species have aimed the development of alternative methodologies to optimize the efficiency of the extraction process.

The development of MAE was first reported in 1986 [22]. During the MAE, the nonionizing electromagnetic radiations heat the extraction medium, transferring the analytes from their cellular environment. In addition, the heat evaporates the water inside the cells, increasing the pressure that increases the cell porosity and breaks cell membranes, to finally improve the solvent penetration [23]. MAE can be developed in open vessels at low temperatures, or in closed systems at high-temperature extractions. The advantage of the microwave is that heating is simultaneously produced in the whole sample, unlike classical conductive heating methods [24]. To achieve an efficient extraction, MAE requires an optimization process in function of the matrix properties, being significant the determination of the solvent-sample ratio, solid-liquid ratios, microwave power, and delivered energy equivalents [25]. This optimization process is performed by the application of experimental design with response surface methodology. MAE has been satisfactorily applied in the extraction of carotenoids from fruits [26], wastes, and by-products [27] or seaweeds [23].

UAE is recognized as the lowest effective method among the nonconventional methods for carotenoid extraction. Consequently, the trend is to combine several *green* methodologies as

MAE and UAE, or to join UAE with solvent extraction methods to enhance the efficiency of the conventional system [28]. UAE utilizes wave sounds of 20 kHz–100 MHz, generating cavitation in the medium through compression and expansion cycles, and consequently, the production of bubbles. The collapse of the bubbles induces disruption of the cell walls and the release of the cellular content. The main benefit of this technique is the extraction at low temperatures.

PLE was developed first time in 1996 [29] and since then it has been successfully applied to different matrices. This methodology utilizes solvents at high temperature and pressure in the ranges of 50–200°C and 35–200 bar, respectively [23]. The high temperatures help in the cell wall disruption process, while the high pressure forces the contact between the solvent and the disrupted matrix [30]. A recent modification introduced in PLE technique is the pressurized hot water extraction (PHWE) allowing the claim of *green* recovery of bioactive compounds from leaves of *Stevia rebaudiana* (Bertoni), with positive results for carotenoids. Nevertheless, the application of different processing conditions including different temperatures (100, 130, and 160°C), static extraction times (5 and 10 min), and cycle numbers (1, 2, and 3) did not improve the carotenoid recuperation [31].

The strategy followed by the PEF is to locate the sample matrix between two electrodes and apply intermittent high electric frequencies with intense electric fields during nanoseconds or milliseconds periods of time. When the transmembrane potential exceeds 1 V, the charged molecules initiate the repulsion, generating pores in the membranes [28], process that could be reversible or permanent. Recently, PEF has been validated as an enhancer of the carotenoid bioaccessibility [32], setting the processing parameters to increase the carotenoid amount incorporated into the micellar phase from tomato fruits.

Since its discovery in 1879 [33], SFE has been applied with a great success mostly at industrial scale. The basis of this technique consists on to leave the substance at temperature and pressure conditions beyond its critical point. At this stage, the supercritical fluid exhibits high diffusivity and low viscosity, features of the gas phase, but develops a high solvation power, a feature characteristic of the liquid phase. Usually, the solvent is carbon dioxide as the critical temperature and pressure are relatively low. SFE can be applied as the traditional *off-line* mode, which means that the extracted analyte is collected in an external collector or in the more recent *on-line* mode, when the extracted analyte is directly transferred to the chromatographic column [34]. Following this approach, it is possible to efficiently extract the targeted carotenoids from red Habanero peppers (*Capsicum chinense* Jacq.) [35]. In comparison with the *off-line* systems, the *on-line* approach seems to improve the run-to-run precision, enables the setting of batch-type applications, and reduces the risk of sample contamination.

The EAE performs the disruption of the cell walls by the activity of different enzymes as pectinases, cellulose, or hemicellulose. Two different strategies are applied: the cell walls and the polysaccharide-protein colloids are the targets of the enzymatic activity of the aqueous-EAE. The alternative method is the cold-EAE, where the enzymes only hydrolyze the cell walls [30].

Independently of the extraction method, sometimes the matrix required a saponification step prior the analysis. Such reaction should be considered as a clean-up process, which eliminates lipids and chlorophylls, facilitating the separation in the HPLC column. On the contrary, if saponification is necessary, it should proceed with caution as several carotenoids are prone to

modification, transformation, or degradation in the presence of KOH. In any case, recently, it has been highlighted the importance of the gap between comprehensive extraction protocols in plant metabolomics studies and method validation [36]. Classically, the determination of specific secondary metabolites has used targeted methods for very specific purposes. But modern metabolomics consist on a data-driven approach with predictive power to determine all measurable metabolites without any preselection. Consequently, classical extraction methods cannot be strictly applied in metabolomics studies and requires validation to new purposes.

3. Liquid chromatography-mass spectrometry

Old fashion methods formerly applied in the analysis of carotenoid, as open column chromatography or TLC are now considered purification techniques, due to its low cost and simplicity. Thus, unambiguously, the HPLC is the most widely used technique in carotenoid analysis currently. Several reviews have been published compiling different HPLC methods for carotenes and xanthophylls [14, 16, 37–40] and specifically for carotenoids esters [15]. Therefore, our aim is to highlight the advantages and limitations of the different alternatives that are available, allowing the reader to evaluate them and select the best option.

Three main sorts of chromatographic columns are applied in the carotenoid analysis: octyl (C_8), octadecyl (C_{18}), and triacontyl (C_{30}) bonded phases. In [41], a high-performance liquid chromatographic method was developed with a reversed phase C_8 column and pyridine-containing mobile phases for the simultaneous separation of chlorophylls and carotenoids. The proposed method also allows the separation of taxon-specific carotenoids belonging to eight algal classes, including some critical pigment pairs for previous HPLC methods. The method works with a binary gradient, so that it can be implemented with both low-pressure and high-pressure mixing instruments. In general, C_{18} columns are used for the analysis of carotenoids with a wide range of polarity as the gradient only requires 10–30 min. To separate and identify geometrical isomers of carotenoids, a C_{18} Vydac 201TP54, one of the more popular columns, is combined with a ternary mobile phase, methanol mixture (0.1 M ammonium acetate), tert-butyl methyl ether, and water [42]. The proposed method allows the separation of 17 different carotenoids, including *cis* and *trans* isomers. Nevertheless, it is generally assumed that C_{30} columns exhibit better resolution for chromatographic separation of the geometric isomers, although the use of this column means more run time (60–100 min) to complete the analysis [43, 44]. Specifically, to control the carotenoids used as food colorants (norbixin, bixin, capsanthin, lutein, canthaxanthin, β-apo-80-carotenal, β-apo-8′-carotenoic acid ethyl ester, β-carotene, and lycopene), there is a method, where the resolution of C_{30} columns allows the intricate chromatographic isolation of bixin and norbixin due to their acidic character [45] in combination with the optimization of accelerated solvent extraction applied to different food matrices. Alternatively, the use of C_{34} columns has also been evaluated, but at least with similar results as with a C_{30} column [46].

The particle size of the stationary phases of HPLC columns commonly used for carotenoid analysis has evolved from the classical 5 μm, through the common 3 μm, finally to reach the more recent sub-2-micron particles. This new technology, known as ultra-high-performance liquid chromatography (UPLC), means that the delivery system of the mobile phases operates

at high back pressure (as an example, HPLC works at 35–40 MPa, while UPLC can reach more than 100 mPa). The advantages of the new UPLC systems are the higher sensitivity, lower run times, and lower consumption of the mobile phases. Years ago, the commercially available columns for UPLC were packed with C_{18}, but in the last 5 years also C_{30} columns with less than 2 μm of particle size are accessible. In Ref. [47], the performance of a conventional C_{30} (3 μm particle size) column with the novel sub-2-micron C_{30} was compared in the analysis of 31 different carotenoids. The results have showed a better compound resolution and shorter analysis time with the novel particle size.

Mono-dimensional (single-column or one-dimensional) chromatography can be considered the most common methodology used for carotenoid analysis. However, a recent development [48] was the application of two-dimensional chromatography to elucidate the carotenoid pattern in citrus. In this technique, the carotenoids are analyzed by two *on-line* chromatographic systems, increasing the separation power and peak capacity [49]. This modality of chromatography should be applied in the analysis of complex matrices or when the carotenoid profile is intricate. The best performance is achieved when both systems operate with complementary selectivity, that is, normal-phase × reversed-phase. A recent optimization implements UPLC in the second-dimensional chromatography increasing the resolution [50]. Even so, the practical performance of this methodology must resolve different challenges including solvent immiscibility or the peak focusing at the head of the second column [49]. To overcome these drawbacks, the normal phase applied in the first-dimensional chromatography could be replaced by supercritical fluid chromatography [35] increasing the identification power. In this sense, a rapid ultra-performance convergence chromatography (UPC^2) method for the determination of seven fat-soluble vitamins (vitamin A: retinol, retinyl acetate; vitamin D: ergocalciferol, cholecalciferol; vitamin E: α-tocopherol; and vitamin K: phylloquinone, menaquinone) and three carotenoids (lutein, lycopene, and β-carotene) was developed [51]. Carotenoids were separated within 3 min with an HSS C-18 SB column (3.0 × 100 mm, 1.8 μm) under isocratic conditions with a mobile phase of carbon dioxide and ethanol (75:25, v/v).

Another parameter to take note is the influence of the injection conditions in the resolution of the chromatography [52]. The authors proposed that the nature of the injection solvent could be responsible of distorted or false peaks. Parameters as the polarity index, solvent strength, injection volume, solute concentration, or solvent selectivity should be optimized before a chromatographic method is developed.

The main solvents utilized as mobile phases in the carotenoid separation are acetonitrile and methanol. The first solvent presents low absorbance in the range of UV light and low back pressure in the column. On the contrary, methanol is less expensive and less toxic [14]. In addition, those solvents are commonly mixed with less polar solvents as dichloromethane, ethyl acetate, tetrahydrofuran, etc., to improve the resolution power. Some methods also include ammonium acetate or trimethylamine to enhance the recovery. Mixtures of those solvents can be applied in isocratic mode, allowing reproducible retention times and relatively stable baselines [39]. As an example, the isocratic HPLC method with a resolution time of 12 min has been developed for the simultaneous detection of eight xanthophylls: 13-*cis*-lutein, 13′-*cis*-lutein, 13-*cis*-zeaxanthin, all-*trans*-lutein, all-*trans*-zeaxanthin, all-*trans*-canthaxanthin, all-*trans*-β-apo-8′-carotenoic acid ethyl ester, and all-*trans*-β-apo-8′-carotenal [53]. The separation

is performed in a YMC C-30 reversed phase column with as a solvent system containing methanol/tert-butyl methyl ether/water. The method can additionally resolve the identification of lutein and zeaxanthin stereoisomers. When isocratic separations are not enough to achieve the separation, then gradient mode elution should be applied. This operation mode improves the sensitivity, increases the resolving power, and allows the elution of retained compounds. On the contrary, the application of gradient mode elution could need the re-equilibration of the column and could give high differences of the detector response [39].

Carotenoids are universally separated through reverse phase HPLC, while scarce methods imply the utilization of normal phase chromatography, and usually associated with the simultaneously detection of compounds of different nature. Thus, a normal phase high performance liquid chromatography method was developed to simultaneously quantify several bioactive compounds in canola oil as α-tocopherol, γ-tocopherol, δ-tocopherol, β-carotene, lutein, β-sitosterol, campesterol, and brassicasterol [54]. The method claims a higher analytical selectivity in less run time that other reported methods.

An additional approach [55] has been the application of software (DryLab) to help in the development of an HPLC method for enhancing the pigments separation. The method consists of the record of retention times of specific carotenoids in four chromatographic conditions, with the aim to predict the retention time in the hypothetical chromatographic method developed by the user (using variables as gradient time, column, column dimensions, and flow rate). With this computer-assistance procedure, the user finally selects the suitable chromatographic conditions to apply. This methodology is highly advisable for the separation of complex carotenoid profiles that phytoplankton species present.

In addition, more than 20 different carotenoids have been identified in blood and animal tissues. The existence of a carotenoid metabolism in animals generates a high diversity of metabolites in the biological samples [38, 40]. Consequently, it is extremely important for the metabolomics studies of the application of hyphenated techniques that could perform the detection of carotenoid metabolites. Recently, it reviewed the analytical methods developed for the identification of carotenoid metabolites in biological samples. Those metabolites are mainly derived from β-carotene, lycopene, lutein, zeaxanthin, astaxanthin, and fucoxanthin [38, 40].

3.1. Mass spectrometry: strategies for ionization and acquisition of mass data

Hyphenated LC-UV-visible-MS instruments have become the standard choice for carotenoid analysis for several reasons. The acquisition of MS data provides structural information that with the sole use of UV-visible detection could not be concluded. Thus, elemental composition can be determined from the measured mass of the molecular/protonated molecular ion with a high degree of accuracy (high-resolution mass or mil mass measurement) to discard alternative compounds that show close values of mass to our target compound, but different elemental composition. Isotopic pattern is a second dimension that can be obtained from the MS data, which may apply as a filtering rule to reject alternative compounds with similar mass but different elemental composition. With both MS data, the number of potential elemental compositions that may fit for each measured molecular/protonated molecular ion is considerably reduced. However, the elemental composition is not enough for the characterization of

carotenoids because several of them share the same elemental composition with different structural arrangements. MS offers a second experimental approach, the tandem MS to obtain fragmentation spectrum with the different product ions that arise from the parent compound. A detailed analysis of the product ions and the operating fragmentation pathways afford the acquisition of crucial evidence for identification.

In addition to the information that MS data produces several drawbacks commonly observed with the application of LC-UV-visible are overcome. MS relieves the identification of co-eluting individual carotenoids or when complex profiles where the UV-visible spectra are not completely resolved by the presence of interfering compounds or when the concentration of the compounds in the sample is low. Another benefit is the multiplicity of instrument configurations (ionization methods and mass analyzers) in combination with different ionization and MS-acquisition modes, which are described below, offering several alternatives to obtain spectra from diverse MS approaches. **Table 1** contains a brief description of several chromatographic methods applied to the analysis of carotenoids in foods and human samples, where the detection system included mass spectrometry.

3.1.1. Ionization methods

The analysis of the target compounds in a mass spectrometer is based on the magnetic and electric fields to apply forces on ions in a vacuum environment. Consequently, a method to provide charged or ionized compounds must be applied to the sample and subsequently produce desorption of those ions into the vacuum system of the mass analyzer. Different ionization methods are available and the main factors to select one of them are the nature of the sample and the aimed information from the analysis. Indeed, some ionization methods are conveniently hyphenated with liquid chromatography, so that the experimental approach and high-throughput screening of complex mixtures are more feasible. Carotenoids are thermally labile compounds and the application of electron impact (EI) and negative ion chemical ionization (CI), which requires vaporization of the sample before the ionization process, presents therefore a main drawback. However, the initial studies in MS of carotenoids applied EI or CI giving the first approaches to the behavior of these compounds under the experimental conditions required for ionization and MS analysis [14, 56–60]. Fast atom bombardment (FAB) is a modification of the EI ion source where a fast atom beam (particles with high kinetic energy) impacts on the target compound dissolved in a liquid matrix (glycerol, thioglycerol, m-nitrobenzyl alcohol, and diethanolamine) yielding molecular ions and fragments. The target compound is maintained at the same temperature of the ion source, usually ambient temperature and therefore, this method widely displaced the EI and CI ionization techniques for the analysis of nonvolatile and/thermally labile compounds. The characteristics of the MS spectra after FAB ionization are related with the properties of the liquid matrix and indeed it produces a chemical background that may interfere with the ions of interest. The successful application of this method hyphenated with liquid chromatography to the MS analysis of carotenoids was reported in [61]. This ionization strategy yields abundant signal of the molecular ion, while scarce fragmentation processes are observed. The use of FAB MS/MS with high-energy collision-assisted dissociation allows the study of the most abundant product ions that contain structural information to confirm the presence of functional groups and the extent of

Sample	Carotenoids determined	Chromatographic conditions	Detection system	Refs.
Serum	Dietary carotenoids	Gradient mode: (A) MeOH/ACN/water 84:14:4 (B) DCM; C30 column (3 μm, 4.6 × 150 mm), flow rate 1 mL/min	PDA/APCI(+)/ Quadrupole	[80]
Fruits and vegetables	Dietary carotenoids and xanthophyll esters	Gradient mode: (A) MeOH/MTBE/water 81:15:4 (B) MeOH/MTBE/water 16:80.4:3.6; C30 column (5 μm, 4.6 × 250 mm), flow rate 1 mL/min	PDA/APCI(+)/ Ion trap	[18]
Human colostrum and mature milk	Dietary carotenoids and xanthophyll esters from MFGM	Gradient mode: (A) MeOH/MTBE/water 81:15:4 (B) MeOH/MTBE/water 7:90:3; C30 column (3 μm, 4.6 × 250 mm), flow rate 1 mL/min	PDA/APCI(+)/ Quadrupole-time-of-flight	[20]
Red blood cells	Lutein, zeaxanthin, α-and β-carotene, β-cryptoxanthin, and lycopene	Gradient mode: (A) MeOH/MTBE/water-3.9 mM ammonium acetate 83:15:2 (B) MeOH/MTBE/ water 8:90:2–2.6 mM ammonium acetate; C30 column (5 μm, 4.6 × 250 mm), flow rate 1 mL/ min	PDA/APCI(+)/ Quadrupole	[102]
Fruits, vegetables, and human plasma	Apo-lycopenals	Gradient mode: (A) MeOH/0.1% aqueous formic solution 80:20 (B) MTBE/MeOH/0.1% aqueous formic solution 78:20:2; C30 column (5 μm, 4.96 × 150 mm), flow rate 1.3 mL/min	PDA/APCI(−)/ Quadrupole-time-of-flight	[83]
Enriched fruit juices	Retinol, retinyl acetate, retinyl palmitate, and β-carotene	Gradient mode: (A) MeOH (B) water; C18 column (5 μm, 4.6 × 150 mm), flow rate 0.9 mL/ min	PDA/APCI(+)/ Ion trap	[103]
Capsicum fruits	carotenes, xanthophylls, and xanthophyll esters	Gradient mode: (A) MeOH/water 82:16:2 (B) MeOH/water 10:88:2; C30 column (5 μm, 4.6 × 250 mm), flow rate 0.8 mL/min	PDA/APCI(±)/ Ion trap-time-of-flight	[104]
Tamarillo fruits	Apo-carotenoids, carotenes, xanthophylls, and xanthophyll esters	Supercritical fluid chromatography: (A) CO_2 (B) CH_3OH; C30 column (2.7 μm, 4.6 × 150 mm), flow rate 2 mL/min and 0.5 mL/min for make-up solvent	PDA/APCI(±)/ Triple quadrupole	[47]
Whole grain flours	Lutein and lutein esters	Gradient mode: (A) MeOH/MTBE/water-10 g/L ammonium acetate 80:18:2 (B) MeOH/MTBE/ water-10 g/L ammonium acetate 6:92:2; C30 column (3 μm, 3 × 150 mm), flow rate 0.42 mL/ min	PDA/APCI(±)/ Ion trap	[21]
Ketchups and Mediterranean soup	Lutein, α- and β-carotene, lycopene, and *cis* isomers of lycopene	Gradient mode: (A) water (B) MeOH (C) MTBE; C30 column (5 μm, 4.6 × 250 mm), flow rate 1 mL/min	PDA/ESI(Li+)/ Triple quadrupole	[65]
Red orange essential oil	Carotenes, xanthophylls, and xanthophyll esters	Two-dimensional liquid chromatography; first dimension, gradient mode: (A) hexane/ butyl acetate/acetone 80:15:5 (B) hexane; cyanopropylsilane column (5 μm, 1 × 250 mm), flow rate 10 μL/min; second dimension, gradient mode: (A) 2-propanol (B) ACN-20% water; C18 column (5 μm, 4.6 × 100 mm), flow rate 5 mL/min	PDA/APCI(±)/ Ion trap-triple quadrupole	[105]

Table 1. Chromatographic methods applied to the analysis of carotenoids in foods and human samples with mass spectrometry detection.

conjugation of the polyene chain [62]. Nevertheless, the group of atmospheric pressure ionization methods (API) has replaced the FAB technique because they show a high versatility and convenience for hyphenation with liquid chromatography. Thus, electrospray ionization (ESI) and atmospheric pressure chemical ionization (APCI) are the ionization methods widely used in the LC-MS analysis of carotenoids [58, 60]. The approach of the API methods consists of a solution of the compounds in a volatile solvent. In the case of ESI, the application of electric potential to the sample in solution transforms it in an electro-sprayed aerosol. Then, a stream of hot gas evaporates the solvent at a high rate to produce gas-phase ions. In APCI, the sample in solution is sprayed at a higher liquid flow than in ESI through a heated vaporizer. The ionization is produced by a corona discharge with ion-molecule reactions taking place at atmospheric pressure. In both cases, the ions are incrementally transferred from the initial atmospheric pressure conditions into the high vacuum at the mass analyzer [63]. This design of the API methods provides the softness feature to the ionization process, increasing the range of compounds to which application of these techniques is possible.

Specifically, ESI requires small amounts of sample and the problems of thermal instability of some carotenoids are avoided [64, 65]. To enhance the efficiency of the ionization process, which takes place in the aerosol, it is frequent that the incorporation of additives to the mobile phase of the LC system, including ferrocene-based derivatives, mildly acidic salts, weakly organic acids, halogen salts, trimethylamine, or ammonium acetate [14, 64–70]. This strategy means a significant advantage in the analysis of carotenes and xanthophyll esters, considering their lower polarity in comparison with the un-esterified xanthophylls. However, the use of APCI for ionization in LC-MS analysis of carotenoids has become general because of its ability over ESI to generate ions from neutrals, that is, carotenes and xanthophyll esters [2, 17, 71–74]. The application of high temperatures in the heated vaporizer may cause thermal degradation of the carotenoids in the source. However, the transfer of the vapor is very fast, so that the sample compounds do not necessarily reach the high-temperature conditions, while the soft collisions that initiate at this stage spend part of the excess energy. APCI allows working in positive ion or negative ion mode, which has demonstrated to be useful to distinguish between some isomeric carotenoids as the deprotonated molecular ions follow specific fragmentation routes [2].

It is relevant to note that the kind of ions produced is related with the ionization method applied. EI and FAB methods yields abundant molecular ions, a quality that is useful for the measurement of the molecular weight. In the case of API methods, positive or negative ions (depending on the ionization mode) are formed, while the characteristics of the solvent and the presence of alkali metal ions trend to produce clusters and adducts. The production of radicals is not frequent in ESI while the APCI method may yield them depending on the actual conditions and the structural arrangement of the analyzed compound.

3.1.2. Mass spectrometers

The mass spectrometer is the final bond in the instrumentation of LC-MS, which may present diverse configurations according to the technology applied for ion acceleration and detection systems. Each configuration performs diverse intrinsic parameters (mass resolving power, mass accuracy, linear dynamic range, and sensitivity) at distinct levels. Consequently, the

hardware is subjected to a constant developing process, in addition to the software capabilities, meeting demanded robustness, reproducibility, and efficiency of the analyses. Accordingly, the experimental parameters associated with these various instrument configurations are related to the capability range that could be reached with the mass analyzer. Indeed, the performance of the instrument is particularly significant to select the suitable strategy for the identification of compounds, as well as to enhance the rigor and strength of the peak assignment. Different mass spectrometers have been applied in the MS analysis of carotenoids, including the ion trap (IT) [18, 75–78] and quadrupole (Q) [79–82] instruments that offer good sensitivity but limited resolving power [63]. These instruments provide mass-to-charge ratio (m/z) values of the molecular ion with mass uncertainty at the part-per-thousand level. The introduction of mass spectrometers with higher peak resolution enhances the rate of mass accuracy measurements and the resolving power of the instrument, allowing the delivering of molecular formula with a lower uncertainty (part-per-million level) as described below. Another significant improvement in the hardware configuration of the mass spectrometer is the incorporation of a second stage of m/z analysis to obtain the mass spectra of selected ions. Thus, the arrangement of mass spectrometers in a tandem configuration to structure hybrid mass spectrometers (Q-time-of-flight, Q-IT, and triple Q-Orbitrap) [2, 20, 64, 83–87] allows the achievement of higher performance by increasing the mass accuracy level and the record of mass spectrum of a precursor ion to generate product ions. This configuration is essential when soft ionization methods are applied because they barely produce fragment ions and the experimental information from the MS analysis would limit to m/z values and isotopic pattern. The application of tandem MS generates a second dimension of data related with the structural arrangements featured in the selected precursor ion for MS/MS analysis.

3.1.3. Experimental information acquired from MS analysis

The primary piece of information that contains a mass spectrum is the m/z values of the ions produced at the ionization source. A pure compound would yield basically only one m/z value corresponding to the molecular/protonated molecular ion (depending on the ionization method applied). The intensity of the signal associated to each m/z value correlates to the abundance of each ion. Alternatively, some product ions could appear at the mass spectrum with smaller m/z values than the parent ion from which they are produced. We have mentioned that this is not usual when soft ionization methods are used, but product ions are necessarily generated in tandem MS equipment, so that a significant increment in the number of m/z signals is observed in the MS spectra generated in such hardware configuration. Hence, the highest m/z value would correspond to the molecular/protonated molecular ion while the rest m/z values are fragments ions derived from the former. As mentioned above, the measured m/z value delivers molecular formula and the higher the accurate level of the acquisition, the lower number of molecular formula corresponding to the m/z value. In the case of carotenoids that usually show m/z values in the range of 450–650 Da, the unequivocal assignment of formula by the measurement of accurate mass is not possible and restrictions, in addition to complementary information from the MS data are required [40]. That information is contained in the isotopic pattern, the second piece of information that should be obtained from MS data, which results from the natural isotope abundance and elemental composition

[88]. The comparison of the theoretical isotopic pattern and the experimental one is a powerful additional constrain for removing molecular formula candidates that initially fitted with a determined *m*/*z* value. To obtain a notion over the matching result of both pieces of analytical information, **Figure 1** depicts the calculated monoisotopic mass, that is, the exact mass of the most abundant isotope, of the protonated molecular ion $[C_{40}H_{56} + H]^+$ (*m*/*z* = 537.4455 Da), one of the possible *m*/*z* values for carotenoids. The experimental MS spectrum in the analysis of a compound yields the *m*/*z* value equal to 537.4462 Da. The application of filtering rules to the mass error and isotopic pattern (error below 3 ppm and isotopic matching value below 50) with a software tool assures the assignment of the $C_{40}H_{57}$ ion formula to the experimental *m*/*z* value, which indeed fits with the starting proposal (see Section 3.2.2).

Although MS data is enough for the identification of several carotenoids without considering additional analytical information (chromatographic behavior, UV-visible spectrum), those with the same elemental composition but different constitution (β-carotene and lycopene), and the isomers require complementary information for the characterization of their structural arrangements because the *m*/*z* values and the isotopic pattern are the same. **Table 2** contains the calculated monoisotopic mass of protonated molecular ions and product ions observed in the analysis of common carotenoids. The aim of **Table 2** is not to compile comprehensive information of the MS behavior of carotenoids, but to remark the reader that different structures share the same molecular weight and fragmentation pathways. Thus, the list of carotenoids with a calculated *m*/*z* equal to 537.4455 Da includes 12 compounds [89]. The comparisons of mass spectra by positive and negative ion modes [15, 90], the possibility of performing additional MS^n experiments of isolated product ions, and even the differences in the intensity of the MS signals are existing alternatives that can aid identification. Among those alternatives, the application of techniques to select *m*/*z* ions, fragment them,

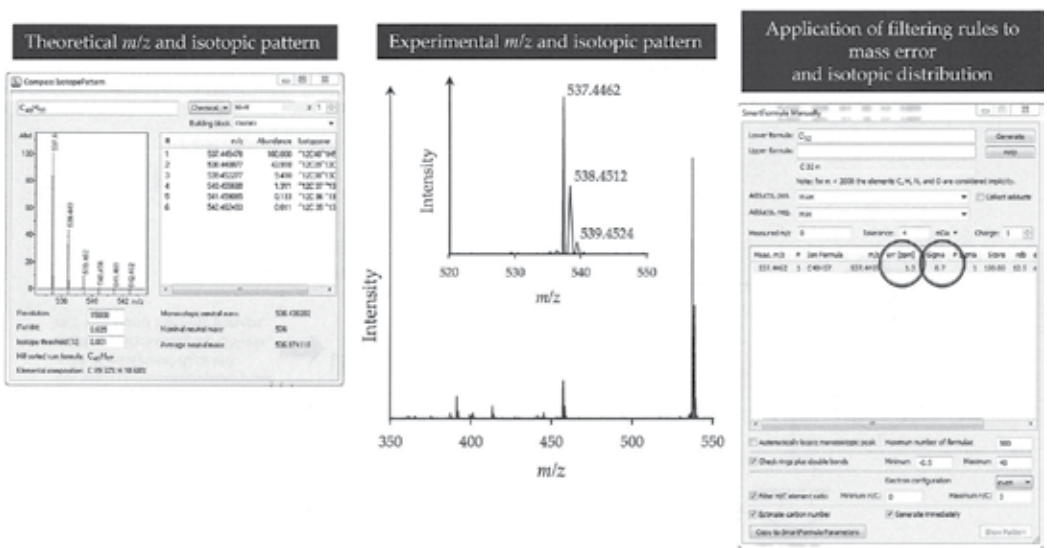

Figure 1. Matching the theoretical *m*/*z* and isotopic pattern with the experimental values and the application of filtering rules for the acquisition of mass ion formula [91, 92].

Carotenoid	Formula	$[M + H]^+$	Product ions		
			In-chain units	Loss from end groups	De-cyclization
α-Carotene	$C_{40}H_{56}$	537.4455	457.3829; 445.3829	413.3203	481.3829
β-Carotene	$C_{40}H_{56}$	537.4455	457.3829; 445.3829	413.3203	—
Lycopene	$C_{40}H_{56}$	537.4455	457.3829; 445.3829	413.3203	467.3673[a]
α-Cryptoxanthin	$C_{40}H_{56}O$	553.4404	461.3778	535.4298	479.3673
β-Cryptoxanthin	$C_{40}H_{56}O$	553.4404	473.3778; 461.3778	535.4298	495.3985
Echinenone	$C_{40}H_{54}O$	551.4247	471.3621; 459.3621	533.4141	495.3621
Lutein	$C_{40}H_{56}O_2$	569.4353	477.3727; 463.3570	551.4247; 533.4141	495.3622
Zeaxanthin	$C_{40}H_{56}O_2$	569.4353	489.3727; 477.3727; 463.3570	551.4247; 533.4141	—
Astaxanthin	$C_{40}H_{52}O_4$	597.3939	505.3313	579.3833; 561.3727	—
Neoxanthin	$C_{40}H_{56}O_4$	601.4251	509.3625	583.4145; 565.4039	—
Violaxanthin	$C_{40}H_{56}O_4$	601.4251	509.3625	583.4145; 565.4039	—

Product ions have been described in [2, 57, 60, 73].

[a]De-cyclization reaction means loss of the acyclic ψ end-group of lycopene.

Table 2. Calculated monoisotopic mass (Da) of protonated molecular ion and product ions observed in the analysis of carotenoids by APCI-MS.

and re-analyze the MS spectrum derive significant information regarding the operative fragmentation pathways while the subsequent analysis of the product ions gives a high amount of data that facilitates the peak assignment and structural characterization. Hence, the fragmentation pathways operating at the molecular ion yield characteristic product ions that allow the identification and location of functional groups, configuration of end-rings, chromophore length, and other key structural features of the carotenoids [2, 57, 60, 66].

3.2. Data analysis and identification

The high amount of analytical information obtained from MS must be conveniently processed to give results with a meaningful biological context, and enough confidence and rigor is demanded when identification is the aim. There is not a single standardized approach for data analysis and identification because the procedure applied to study the MS spectra depends on the chemical class of the analytical compounds and the instrumental techniques used to obtain the data, so that diverse workflow models are possible. The acquisition of experimental data from hyphenated LC-UV-visible-MS derives a minimum of three dimensions on the information: chromatographic behavior or retention time, UV-visible spectrum, and MS spectrum of each chromatographic peak that should correspond to a molecular/protonated molecular ion. An additional dimension of information is obtained when tandem MS experiments are performed with the selected precursor ions. Therefore, several independent and complementary physicochemical properties are available (chromatographic behavior,

http://dx.doi.org/10.5772/intechopen.79755

UV-vis spectrum, *m*/*z*…). The key in the data analysis of LC-UV-visible-MS data is to organize the physicochemical features on pairs of independent and orthogonal data (retention time and mass spectrum, accurate mass and tandem MS, and accurate mass and isotopic pattern). Once, the information is organized, this could be compared with the same analytical pairs of data relative to authentic compounds (standards) analyzed with the same identical experimental conditions [9] to match the data in the sample with those of the standards. This strategy is summarized in **Figure 2** and involves the following advantage. The process of matching the experimental data of the sample and those of the standards for the selected orthogonal criteria could be automated for reporting of findings by means of post-processing software tools [91, 92]. Among the different pairs of possible orthogonal data, accurate mass and isotopic pattern are widely accepted to successfully characterize the elemental composition of compounds in combination with orthogonal data related with the chromatographic behavior and UV-visible features. Thus, in the analysis of a complex mixture of carotenoids by LC-UV-visible-MS, a list of compounds with different *m*/*z* values and isotopic pattern is obtained, and a first matching process among the experimental data and the standards could be performed. This first screening is not unequivocal, because for each single pair of *m*/*z*

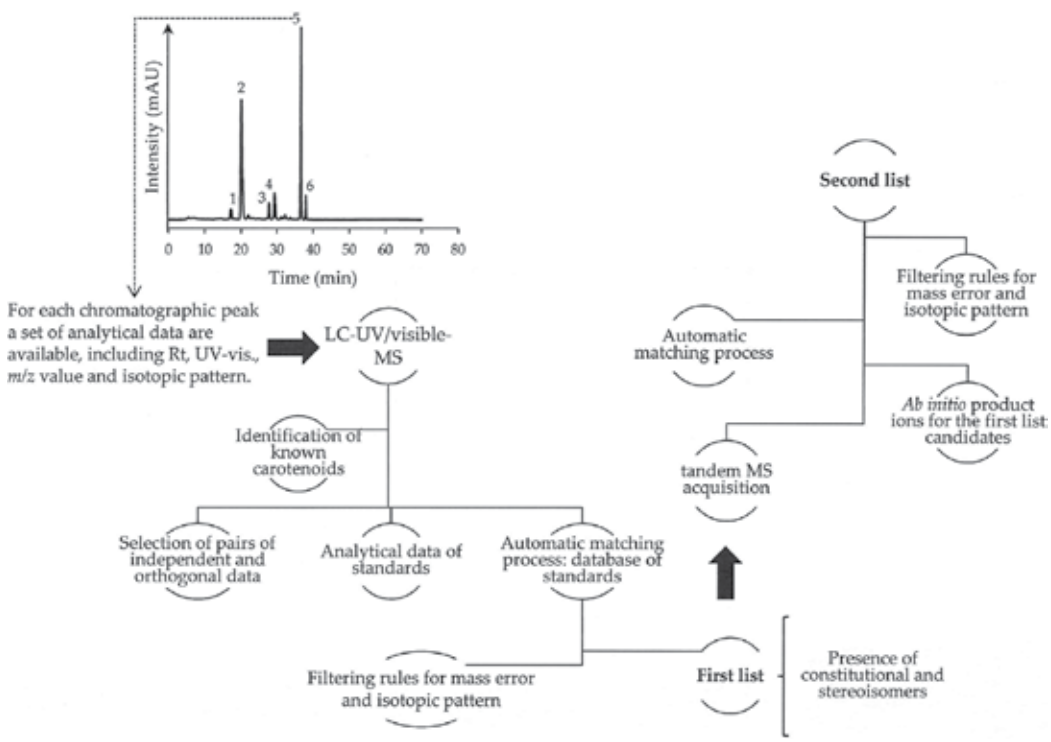

Figure 2. Overview of the workflow model for data analysis and identification of carotenoids. The selected pairs of independent and orthogonal data are matched with the corresponding experimental values of standards acquired with the same conditions. A first list of tentative identified carotenoids is obtained. This list is not unequivocal, and subsequently tandem MS data are obtained to match the experimental values of product ions with the *ab initio* product ions produced with the assistance of predictive software tools. The second list of identified carotenoids fulfills the rules and requirements for validating the identification [9, 40].

and isotopic pattern values a second reduced list of alternatives is possible due to the possible presence of constitutional and stereoisomers compounds in the sample (**Figure 2**). For example, it is not possible to distinguish β-carotene from lycopene or lutein from zeaxanthin exclusively with the *m*/*z* and isotopic pattern data (**Table 2**). In the first case, the assignment could be constrained considering the chromatographic behavior and UV-visible spectrum, but still the assignment is not explicit and for stereoisomers further information is needed. Here is where the third dimension of information plays a key role. The tandem MS data may contain characteristic product ions that arise from a fragmentation route available for one of the isomers and not for the others. Therefore, a list of potential "qualifiers" product ions could be built *ab initio*, which contains their *m*/*z* and isotopic pattern pair of values. This database is matched with the experimental tandem MS data acquired for the target compound to screen whether the "qualifiers" product ions are present or not (**Figure 2**). The search for these "qualifiers" product ions starts not only with the acquisition of tandem MS spectra, but also with the application of software tools to predict their structure considering the knowledge of the fragmentation rules that may be applied for each compound (see Section 3.2.2).

3.2.1. Common fragmentation pathways of carotenoids

The acquisition of tandem MS spectra encompasses a two-stage mechanism with a single aim, to produce an ion with enough internal energy (first stage) that causes it to fragment and generate a mass spectrum (second stage). The first stage starts with the ions produced at the ionization source, which are activated by collision with a neutral species. The transfer of energy from the neutral species may result from different processes [93] that are an extensive field of study regarding the characterization of the collisional-activated ion structure [94]. The second step consists on the competitive unimolecular dissociation routes that the collisional-activated ion experiments. According to the theory of unimolecular reactions, the Rice-Ramsperger-Kassel-Marcus theory, the dissociation of a sufficiently large and suitably energized molecule is basically statistical, that is, the internal energy of the activated ion is statistically distributed among the internal degrees of freedom [95]. Consequently, different energetically accessible dissociation/fragmentation pathways start to compete yielding a mass spectrum containing different product ions. The key in this second step is that once the structure of the collisional-activated ion is known, the sequence of fragmentation pathways can be modeled, and the structure of the product ions are predicted [96, 97]. This results in a significant advantage for the study of tandem MS spectra because, as it was mentioned above, the product ions from a selected molecular/protonated molecular ion could be known *ab initio* and the screening in an experimental tandem MS spectrum could be automatically performed with the aid of the appropriate software tool (see Section 3.2.2).

The carotenoids follow three major fragmentation processes: elimination of in-chain units, elimination of terminal groups, and *retro*-Diels-Alder fragmentation [56] (**Table 2**). Indeed, several mechanisms are possible within each of the noted fragmentation schemes, because the different localization of functional groups in the structure of carotenoids, and the frequent stereochemical arrangements introduce alternative events in the progress of the fragmentation process. These mechanisms have been extensively reviewed in [2, 56, 57, 60] with tabulated data regarding the main product ions and the structural significance of the observed

losses and the intensity of the signal in the parent compound. The ionization technique and the composition of the mobile phase are factors that may modulate the fragmentation pattern, but the characteristic product ions are observed under different experimental conditions and instrumental configurations.

The elimination of in-chain units follows the Woodward-Hoffmann rules and the process yields to the losses of toluene (92 Da) and xylene (106 Da) from the polyene chain. Both losses are almost ubiquitous in the MS spectra of carotenoids, and the intensity ratio for the corresponding peaks is inversely related with the number of conjugated double bonds, providing information about the nature of the polyenoic chain [98]. Indeed, the kinetics of the reactions leading to both fragments is modified not only by the amount of double bonds of the chromophore system, but also by the cyclic/acyclic conformation of the end groups and the presence of oxygenated functions. Moreover, the ionization method applied changes the abundance of the signals as it is the case of APCI [60, 66]. Therefore, the information obtained from the intensity ratio should be carefully analyzed as the influencing factors are multiple. Other reactions lead to alternative product ions by the loss of 78, 79, and 80 Da from the parent compound, involving functional groups close to the polyenoic system like the 5,6- and 5,8-epoxides. The analysis of the product ions arising for the elimination of terminal groups represents a helpful diagnostic strategy to establish the structural arrangements and functional groups that feature the carotenoid conformation. The loss of 69 Da indicates the presence of at least one linear ψ end group [73, 99]. The loss of 18 Da points to the presence of hydroxyl group(s), while the loss of fatty acid(s) shows the occurrence of xanthophyll esterification [15, 17, 73]. Some of these product ions reveal further structural information when the signal intensity is accounted. Thus, the loss of 18 Da detected in the MS spectrum of lutein and zeaxanthin shows different abundance (higher for lutein, lower for zeaxanthin) in comparison with the signal of the molecular/protonated molecular ion [60, 73, 100]. A similar behavior displays the pairs of structurally related carotenoids like α-cryptoxanthin and zeinoxanthin or lutein epoxide and antheraxanthin for the same signal (loss of 18 Da) in comparison with the intensity of the molecular/protonated molecular ion [73, 101]. The reactions based on the *retro*-Diels-Alder fragmentation are the third general scheme to follow the interpretation of tandem MS of carotenoids. The reaction may proceed in a single step or by a double α-cleavage starting at the allylic position from where subsequently the fragmentation is produced. This process yields characteristic product ions that can be used to distinguish isomeric α-carotene, β-carotene, γ-carotene, and lycopene, in combination with other characteristic product ions arising from alternative fragmentation pathways. Hence, the *retro*-Diels-Alder fragmentation at the ε-ring of α-carotene generates the loss of 56 Da, so that its presence or absence is indicative of the configuration at least in one of the cyclic end groups [60, 73]. The same applies to distinguish lutein, which presents one hydroxylated-ε-ring and one hydroxylated-β-ring, and zeaxanthin that includes two hydroxylated-β-rings. The product ion at 495 Da that generates from the *retro*-Diels-Alder fragmentation of the ε-ring (56 Da) and subsequent water loss (18 Da) is observed in lutein but not in zeaxanthin [2, 62, 82].

The occurrence of these fragmentation processes with different prevalence (some product ions may not appear, while other could constitute the base peak of the MS pattern) produces a fragment MS spectrum that should provide enough information for the characterization of the different carotenoids present in the sample. The application of published data on the

characteristic MS spectrum of carotenoids and the search in databases on the Internet is helpful for interpretation, but they should be applied with caution considering the instrumental configuration and conditions for acquisition of the MS that were used in the references, to avoid confusing or incorrect identification. The use of experimental data from authentic standards analyzed with the same MS acquisition settings used for the sample, as well as any complementary source of data should be considered a golden rule for identification.

3.2.2. Software tools

The implementation of software tools to assist in the production of a positive list of identified compounds in sample is one of the significant advances that has been introduced in the capabilities of MS. The analysis of MS spectra for the identification of the molecular/protonated molecular ions, the application of filtering rules based on the mass accuracy error and isotopic pattern, and the study of product ions in tandem MS means an intensive manual labor that could be released with the aid of suitable software. These tools may be embedded as a module or option in the main software distributed with the MS instrument, or may be available to download from the Internet. Different software utilities are designed to resolve each of the steps that lead to obtain the correct elemental composition and to establish the main structural features.

To achieve that aim, the application of filtering rules to the measured mass, regarding the mass error and isotopic pattern distribution, is the main approach to constrain the number of possible candidate structures. Thus, for a given molecular/protonated molecular ion with an experimentally measured *m*/*z* value, a set of elemental compositions is possible (see Section 3.1.3 and **Figure 2**). Increasing the mass accuracy, the number of potential candidates is reduced. The software tools to perform this first screening automatically, the operator should only establish the mass error value to allow an elemental composition to enter in the positive list or to be excluded. The second filtering rule involves isotopic pattern. The software tools compare the isotopic distribution of the experimental *m*/*z* value with the theoretical ones of the potential candidates that fulfilled the first filter (mass error) yielding a correlation value for each candidate. The operator should only establish the threshold for that correlation value that makes an elemental composition to pass this second screening. Free software to complete both screening steps is available on the Internet (http://tarc.chemistry.dal.ca/soft_down.htm; https://omics.pnl.gov/software/molecular-weight-calculator). Regarding the evaluation of data from tandem MS spectra, the same programs may apply to each of the characteristic product ions. The filtering rules process should be reliably accomplished, both in the parent compound and in its product ions. Thus, the consistency of the elemental composition and isotopic pattern of the product ions with the structural features of their parent compound is assured [91, 92]. To perform this step and the subsequent study of the contribution of each characteristic product ion to the structure of the molecular/protonated molecular ion, we should be able to predict product ions from the candidate structures ascertained in the first screening process. As it was noted in Section 3.2.1, the product ions generated in a tandem MS spectrum could be modeled by the application of the general fragmentation rules. Predictive software tools (Mass Frontier, HighChem, Mass Fragmenter, Advanced Chemistry Development, and EPIC) evaluate the starting structure of the parent compound and generate a set of hypothetical product ions that may arise from fragmentation. This list of candidate product ions could be matched with the list of experimental

product ions observed in the tandem MS spectrum. Once the annotated product ions are tabulated, the filtering rules regarding mass error and isotopic pattern assure that the assignation of product ions is unambiguous and related to the parent compound. Indeed, this second screening process contributes to discard some of the candidate structures or even to point only one of them as the assigned structure for a given molecular/protonated molecular ion.

4. Conclusions

The advance in knowledge of carotenoids occurrence in nature has required the application of different analytical techniques to characterize the structure of this family of pigments, how they accumulate in different tissues and the available metabolic conversions they experiment either in their natural cellular surrounding or in the tissues they are incorporated. Classical procedures for the extraction of carotenoids from raw sources and different biological materials have evolved with the inclusion of *green* technologies and environmentally friendly practices, which are summarized in this chapter. Indeed, the development of HPLC methods has introduced the new generation of stationary phases that shortens the run time and solvent expenditure. With the application of UPLC and two-dimensional liquid chromatography, the challenge of the characterization of intricate carotenoid profiles has been accomplished, and it is possible to select almost tailored chromatographic conditions to the target sample matrix containing the carotenoids. Thus, a relevant improvement has been the analysis of xanthophyll esters, which was a difficult approach few years ago. The current accessibility to mass spectrometry detectors has overcome several of the drawbacks that the traditional detection/identification of carotenoids by the UV-visible spectrum. This has been possible due to the improvements in the ionization strategies and equipment applied to yield ions mass, as well as the use of mass spectrometers with high capabilities in terms of mass resolving power, mass accuracy, linear dynamic range, and sensitivity. Thus, the acquisition of full detailed MS data in conjunction with the information obtained from the chromatographic behavior and UV-visible features produce the accurate evidences for the correct identification and provide the appropriate biological meaning to the research issue. Finally, the practice in software tools implemented in the workflow of MS data analysis alleviates the manual labor of data processing and allows the systematic high-throughput screening of carotenoids and their metabolites.

Acknowledgements

The financial support of the Spanish Government (Ministerio de Economía y Competitividad, projects AGL2015-63890-R and AGL2017-87884-R) is gratefully acknowledged.

Conflict of interest

The authors declare no conflict of interest.

Author details

Antonio Pérez-Gálvez* and María Roca

*Address all correspondence to: aperez@ig.csic.es

Food Phytochemistry Department, Instituto de la Grasa (CSIC), Campus Universitario, Sevilla, Spain

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Chapter 3

External Field Effect on Electronic and Vibrational Properties of Carotenoids

Nan Gong, Zuowei Li, Chenglin Sun and Zhiwei Men

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.78593

Abstract

Resonance Raman spectroscopy is one of the most popular molecular spectroscopy methods. Using this technique, carbon-carbon (CC) vibration can be well investigated and give much information on π-conjugated system. The CC vibration has a strong dependence on the π-electron band gap, which is able to be characterized by absorption spectroscopy. Electron-phonon coupling will strongly influence the Raman intensity and shows sensitivity to the surrounding environment. Through electron-phonon coupling, CC vibration can be modulated by the π-electron band gap. In this review, we present our absorption and Raman-scattering observations of all-trans-beta-carotene from different environment such as temperature, pressure, solvents, and phase transition. The relationship between π-electron band gap and CC vibration is studied. With the decreasing of the π-electron energy, the modulation on CC vibration and electron-phonon coupling is enhanced leading to spectral red-shift. This review has significant importance in understanding the resonance Raman scattering, structural properties of polyenes, and the physics behind it. The results are also valuable for the development of fine-quality photoelectric device.

Keywords: carotenoids, absorption shift, Raman scattering cross section, electron-phonon coupling

1. Introduction

Carotenoid pigments are found in organisms from many phyla and in all wild-type photosynthetic bacteria, algae, and higher plants examined to date [1, 2]. They are important components of biomimetic photosynthetic constructs where much of the photophysics and

photochemistry including energy transfer, electron transfer, radical pair recombination observed in natural photosynthetic reaction centers, and antennas can be mimicked [2, 3]. They also have a protective function against the drastic change of the environment, that is, the dissipation of excess energy. Molecular properties of carotenoids are sufficiently studied using multiple methods. Understanding the intermolecular interactions is one of the primary objectives. Correlating the intermolecular interactions with physical properties in a condensed phase will help in interpreting the structure dependence of carotenoids in different environments [3, 4]. The all-trans-β-carotene (β-Car), with 11 conjugated double bonds, has unique biochemical and optical properties and is considered to be a model in studying conjugated polyenes. Conjugated polyenes are electroluminescent materials that are well studied because of their significance in both physics and chemistry [5–9]. This fact, combined with their functional properties of semiconductor and their solubility in common organic solvent, makes the conjugated polyenes a new class of electronic plastic materials with potential applications in electro-optical and photovoltaic devices. Many organic thin films have been fabricated and their physical properties have been examined in order to develop fine nonlinear optical devices. Carotenoids, although due to their poor structural stability, can be a good model to investigate because of their large nonlinear optical susceptibilities [9–12]. Different types of β-Car thin-films were fabricated, and their optical absorption and resonance Raman spectroscopy were examined. Photoinduced and time-resolved absorption spectroscopy reveal that the infrared absorption bands may correspond to the recombination of the soliton-like excitations in β-Car single crystals [13–16]. Spectroscopic properties and dynamics of the excited singlet states have been investigated in different liquids or polymers as a function of refractive index [17], static permittivity [18], temperature [19], pressure [20], and external electric field [21–23]. Although the solvated carotenoids have been extensively studied, the temperature dependence of Raman-active modes of carotenoids remained unknown until the work by reference [24]. The results suggested that the spectral properties of CC-stretching modes are very sensitive to the temperature, which was similar to that of polyconjugated molecules. In addition, they revealed that the temperature effect and vibronic coupling together contributed to the observed Raman mode shifts. The position of v1 Raman band versus conjugation chain length was revealed by combining the use of electronic absorption and Raman spectroscopy [25]. Carbon disulfide and pyridine have been chosen as solvent because of their polarizability, which are close to that of membrane lipids and proteins [26]. Observations based on different laser excitation of β-Car provided strong evidence that the enhancement of the Raman bands should be explained by electron-phonon coupling mechanism [27]. Another model, named coherent weak-damping CC vibration model, also indicated that the unusual strong intensity enhancement of polyene's overtone bands should be contributed by π-electron-phonon coupling [28]. Recent resonance Raman spectroscopic study in vivo concluded that the use of a high polarizability solvent was necessary to mimic the electrostatic environment in vivo. However, more experiments are needed on excited-state and ground-state dynamics dependence on environmental effect. Therefore, the external effects on electronic absorption and Raman scattering should be investigated and electron-phonon coupling strength in different external fields should also be examined. In this review, temperature, pressure, solvent polarizability, and phase transition effect on CC vibration modes and electron-phonon coupling are studied. The results reveal that the electron-phonon coupling can be deduced by

using the electronic band gap and Raman intensity. Different mechanisms are introduced to explain the external field dependence on β-Car electronic and vibrational properties.

2. Electronic and vibrational properties of carotenoids

β-Car has nine CC double bonds (C=C) and single bonds (C–C) and is a typical model of π-electron system. The work in early 1970s by references [29, 30] proposed that the lowest-lying excited state $S_1(2^1A_g^-)$ was silent in absorption spectrum. The most likely reason is that the Franck-Condon factors for a vertical transition are negligible since the final state is massively displaced [31]. The strong absorption of β-Car happened from π-π* transition, which is also called $S_0(1^1A_g^-)$-$S_2(2^1B_u^+)$ transition. The excited S_2 state decays by internal transition to S_1, and itself decays to ground state S_0 in the same way. Recently, other "dark" states S* have been proposed for relaxation pathways in several computational chemistry [32]. The S_0–S_2 transition displays a characteristic three peak structure by promoting a single electron from its ground state to the lowest allowed excited state which are termed 0–0, 0–1, and 0–2 (**Figure 1**). Resonance Raman scattering is ideally vibrational technique for observation on carotenoids electronic ground state. Four main bands are observed in resonance Raman spectrum. **Figure 2** shows the resonance Raman spectrum from β-Car, four main fundamental bands are labeled from v_1 to v_4. The most intense band is v_1 from C=C vibration, which depends strongly on π-electron conjugation and molecular configuration. The v_2 bands arise from C–C bands stretching coupled with C–H in-plane bending modes [34]. The v_3 band around 1000 cm^{-1} is assigned as in-plane rocking vibrations of the methyl groups and v_4 band near 960 cm^{-1} arises from C–H out-of-plane wagging motions

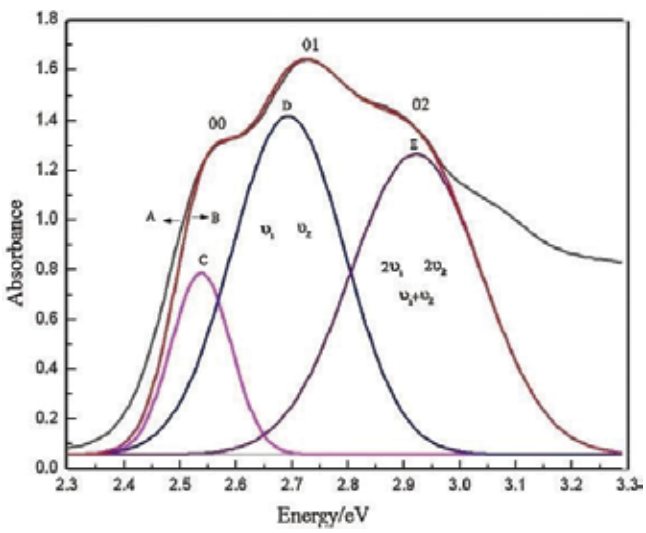

Figure 1. Absorption of β-Car dissolved in cyclohexanol. Line a represents the experimental curve and B is the fitting curve. Components C, D, and E are Gaussian deconvolution of the experimental curve (see Ref. [35]).

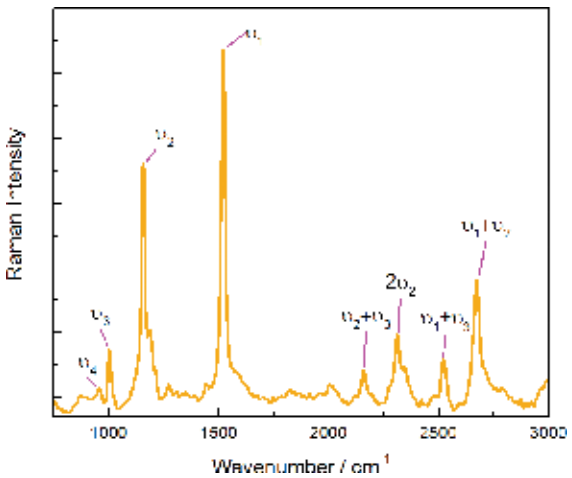

Figure 2. Raman spectrum of β-Car dissolved in carbon disulfide recorded at the room temperature. The fluorescence background has been subtracted.

coupled with C=C torsional modes. The overtone $2v_1$ (2310 cm^{-1}), $2v_2$ (3040 cm^{-1}), and combination $v_1 + v_2$ (2675 cm^{-1}) are also appeared in the resonance Raman spectrum.

3. External fields effect on electronic and vibrational spectroscopic properties

3.1. Temperature dependence on the optical properties of β-Car

There are many researches on electronic and vibrational properties on polyene. Even though the main goal of the extensive studies on polyene properties is to develop optic-electronic devices working in room temperature, studies focusing on temperature dependence of photophysical properties can provide valuable information about the electronic and vibrational characters of conjugated polyene materials. It is essential to have a profound knowledge on excited and ground states of β-Car in order to understand the mechanism of the photoprotective and antioxidative function of carotenoids. It is known that the steady-state absorption and photoluminescence spectra of inorganic materials usually red-shift with the increasing temperature [33, 35]. Extensive temperature dependence researches on conjugated polymers can provide valuable information for studying the β-Car optical response in low temperature. The spectral shift of conjugated polymers can attribute to the thermal conformational changes, which is mainly due to the change of their effective conjugation length. Using the relationship between the zero-phonon and vibrational peak intensities, we can obtain the Huang-Rhys factor. This is an effective parameter which can describe the electron-phonon coupling of the polymer material in different temperature. Given that the unexpectedly high Raman scattering activity of CC bond length vibrations is due to the extended π-conjugation giving a strong electron-phonon coupling, the π-electron delocalization and the electron-phonon coupling should show dependence on temperature.

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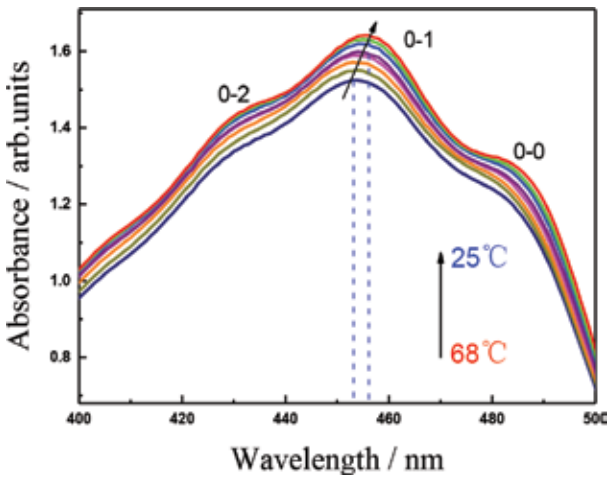

Figure 3. Absorption spectra of β-Car dissolved in cyclohexanol in different temperatures from 68 to 25°C.

We measured the absorption of β-Car dissolved in cyclohexanol in the temperature range from 68 to 25°C with an interval of 5°C. As shown in **Figure 3**, with the decreasing of the temperature, the thermal motion, the entropy of the molecule decreases and the thermal disorder of the β-Car molecule decreases. At the same time, the degree of order of the molecular structure increases, the molecule becomes straight, so that the π electron delocalization expands. The π-π* transition energy narrows which leads to the observed absorption redshift, which is different from the absorption properties of inorganic materials. With the narrowing of the π-π* gap, the influence on the CC vibrational bands from electronic transition becomes stronger. Correspondingly, we recorded the resonance Raman spectra in the same temperature range in order to examine the influence from the narrowing π-π* gap. **Figure 4** shows the resonance Raman spectra of β-Car molecule dissolved in the cyclohexanol in different temperature from 60 to 20°C with the 514.5-nm laser excitation. The bands' frequency

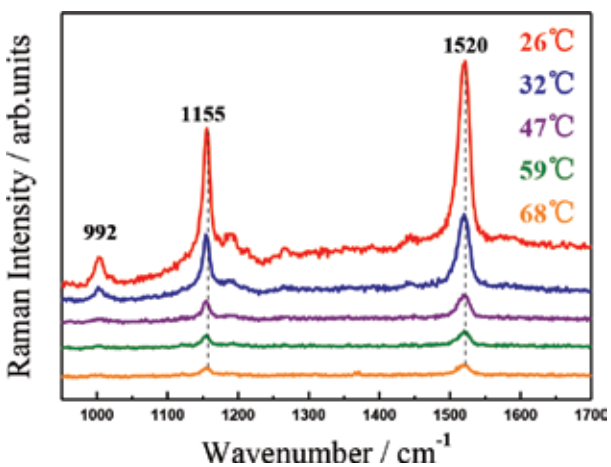

Figure 4. Resonance Raman spectra from 68 to 26°C of β-Car dissolved in cyclohexanol. The laser excitation wavelength is 514.5 nm.

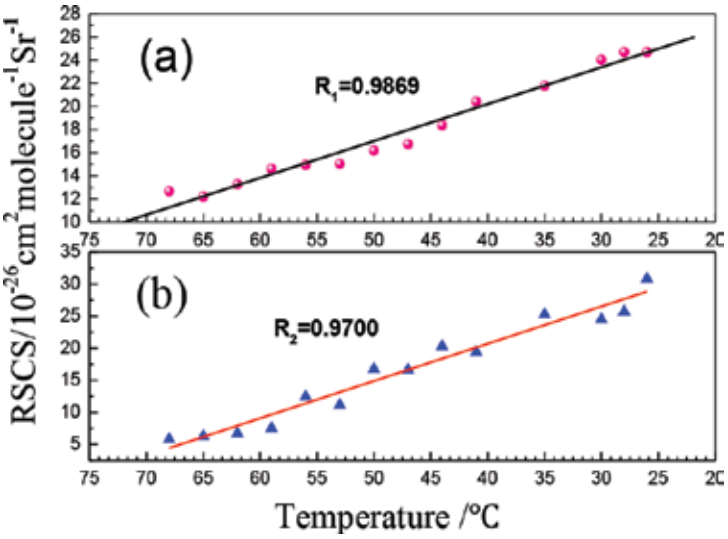

Figure 5. Temperature dependence of RSCSs from (a) C–C and (b) C=C vibrations.

present little shift in this small temperature range, which is consistent with the work by [24]. However, the intensity variation from the resonance Raman scattering results is relatively large and more obvious. Taking the advantage of the Dudik equation, we can calculate the C=C and C—C modes Raman scattering cross section (RSCS). From **Figure 5**, with decreasing temperature, the RSCS declines, which means that the π electron modulation on CC vibration is strengthened. Finally, the observed Raman intensity enhances.

In order to get an evaluation on electron-phonon coupling strength, we use the method reported by [36] to calculate the electron-phonon coupling constants and their temperature dependence which can also reflect the effective conjugation length. The equations can be expressed in the simplified form as follows:

$$S = \frac{I_{10}}{I_{00}}, \tag{1}$$

$$S = \frac{V_1^2}{\omega_1^2} + \frac{V_2^2}{\omega_2^2}, \tag{2}$$

$$\frac{I_1}{I_2} = \frac{\left(\frac{V_1^2}{\omega_1^2}\right)}{\left(\frac{V_2^2}{\omega_2^2}\right)}, \tag{3}$$

where S is the Huang-Rhys factor, I_{10} and I_{00} can be extracted from absorption spectrum, which, respectively, represents the zero-phonon line intensity and the first vibrational peak intensity. The V_1 and V_2 are the electron-phonon coupling constants, which lead to the broadening of absorption band and resonant enhancing of the two totally symmetric phonons observed in Raman scattering

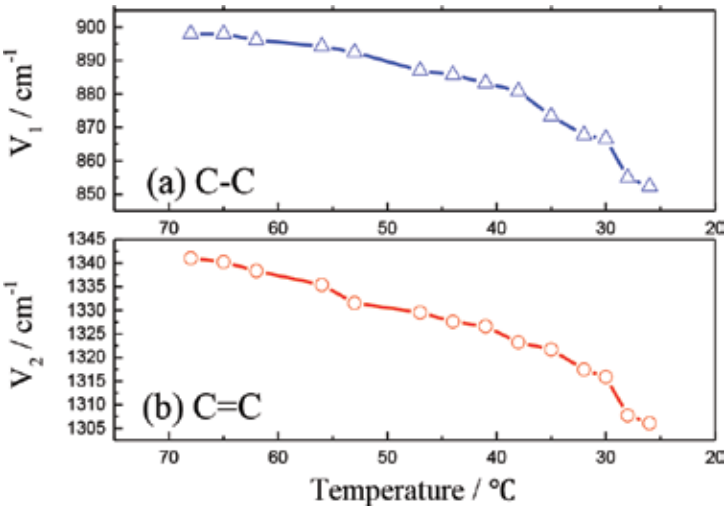

Figure 6. Temperature dependence on (a) C—C and (b) C=C electron-phonon coupling coefficient.

(ω_1 = 1155 cm^{-1}, ω_2 = 1520 cm^{-1}). With the I_1, I_2, and ω_1, ω_2 obtained by Raman spectra, the V_1/ V_2 could be calculated. Combining this result with the value of HR factor from absorption spectrum, it is then allowed to derive the electron-phonon coupling constant for the dissolved β-Car molecule.

The results are shown in **Figure 6**, which indicate that the modulation on CC modes becomes stronger and the electron-phonon coupling is enhanced. As a result, the CC modes show Raman intensity enhancement.

3.2. Pressure dependence on the optical properties of β-Car

Pressure dependence of absorption and resonance Raman scattering of β-Car was pioneered by [37] in the 1980s. Their research firstly showed that the absorption from β-Car under pressure presented a large red-shift and a strong broadening of the vibronic peaks. This work suggested that the assumption of linear coupling of pressure to configurational coordinate needed to be modified and the potential energy curves showed pressure-induced shift. Better information about this was reported later by their works on resonance Raman scattering of β-Car. However, they concluded that the configurational coordinate models and solvent models could not sufficiently explain the large shift in the electronic spectrum of β-Car under pressure [38]. The work by Ref. [39] studied the solvent effect and pressure effect on β-Car dissolved in n-hexane and carbon disulfide. It was concluded that the spectral response from β-Car should due to the environmental effect rather than structural distortion and considered high-pressure technology as a potential way in exploring the biological functions of carotenoids.

The absorption and resonance Raman spectra of β-Car molecules at pressures of 0.04–0.60 GPa in carbon disulfide were measured. The experimental result (**Figure 7**) is that as the pressure increases, the absorption spectrum red-shifts and the reason should be the fact that the molecules are compressed and the π-electron energy gap decreases under pressure. The CC bond length is shortened, and the π-electron delocalization is blocked and thus the energy increases.

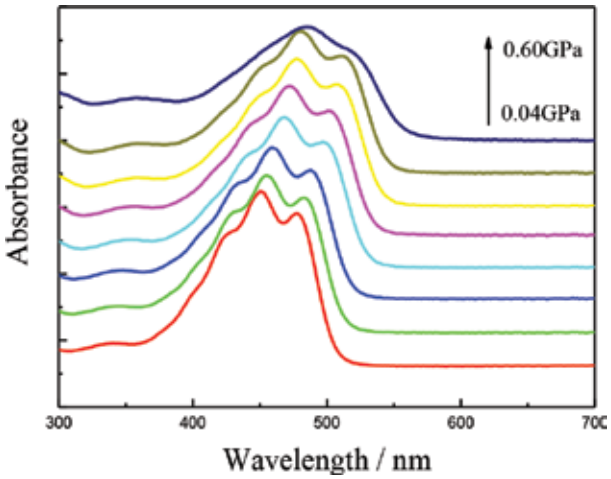

Figure 7. Absorption spectra from β-Car dissolved in carbon disulfide under pressure from 0.04 to 0.60 GPa.

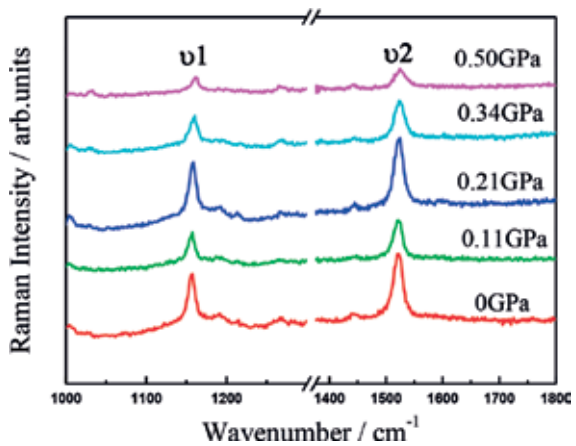

Figure 8. Raman spectra of C—C (1155 cm^{-1}) and C=C (1520 cm^{-1}) bond in different pressures.

It is evident from **Figure 8** that as the pressure increases, the Raman spectra blue-shift and the intensity of the Raman spectra decreases with increasing pressure. Obviously, the absorption spectra and Raman spectra changes are different very much from the effects of temperature on β-Car molecules. When the temperature is lowered, the absorption spectrum and the Raman spectrum are red-shifted, and the spectral intensity is increased. This is due to the π-electron energy gap that enhances the modulation of the CC bond vibration. Electron-phonon coupling enhancement is perfectly explained by theories such as "effective conjugate length" and "coherent weakly damped vibration" of linear polyene molecules. When under pressure, the absorption spectrum of β-Car is red-shifted, the Raman spectrum is blue-shifted, and the Raman intensity is reduced. The above theory cannot be explained satisfactorily. The

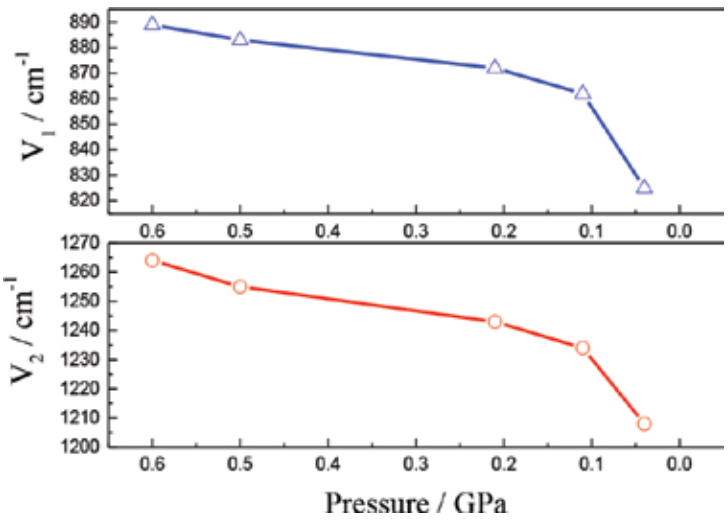

Figure 9. Pressure dependence on electron-phonon coupling coefficient V_1 and V_2.

formation of the resonant Raman spectrum based on the linear polyene molecule is the result of the vibrational modulation process of the π-electron energy gap on the CC atom, which is the result of electron-phonon coupling. Similarly, according to **Figures 7** and **8**, using theory reported by [36], it is calculated that as the pressure increases, the electron-phonon coupling coefficient increases (**Figure 9**), that is, the electron-phonon coupling strength decreases. The π-electron energy gap weakens the modulation of the CC bond vibration. It is thus able to conclude that as the pressure increases, the β-Car molecule is compressed, the energy of the system increases, and the π-electron energy gap decreases. Therefore, the π-electron modulates the CC vibration, the Raman spectrum blue-shifts, and the intensity decreases.

3.3. Solvent effect on the optical properties of β-Car

The polarizability, polarity, density, and dielectric constant of the solvent will affect the system energy and energy gap of the π electron and thus influence the π-electron energy gap modulation on CC vibration [40]. The absorption spectra and resonance Raman spectra of β-Car in 10 different polarizability solvents were measured. As the polarizability increases, the absorption spectra of β-Car red-shifts (**Figure 10**).

This is because when the β-Car molecule is in the vibrational ground state, two π electrons in C=C are in the π bond orbital, and there is no polarity. When the π-π* transition occurs, π electrons are in the π-bonded orbitals and the π* anti-bond orbits, respectively, and lead to generate polarities. The excited state polarity is stronger, so that the energy drop is greater than the ground-state energy. Therefore, the difference between the π electron energies becomes smaller. At the same time, as the polarizability increases, the concentration of the solution density increases, the space of the β-Car molecule swing decreases, and the order of the molecular structure increases. In this context, the effective conjugate length increases.

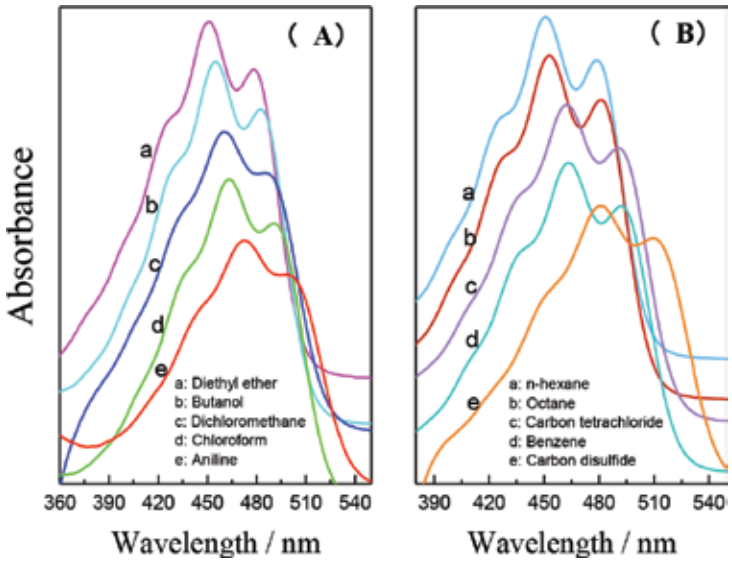

Figure 10. Absorption spectra of β-Car in (a) polar solvents and (B) non-polar solvents.

The reduction of the π-electron energy gap causes red-shift of the electron absorption peak. **Figure 11** shows the resonant Raman spectra of measured C═C, C=C modes of β-Car molecules. Extracting data from **Figure 11**, it can be calculated that as the solvent polarizability increases, the Raman scattering cross section of the CC bond increases. The increase in the polarizability and the increase in the molecule induced dipole moment.

According to **Figures 10** and **11**, using the method of calculating electron-phonon coupling constant by [36], the variation law of electron-phonon coupling coefficient with solvent polarizability is obtained (**Figure 12**). It is able to conclude that as the polarizability increases, the π-electron energy gap enhances the modulation of the CC bond vibration, the electron-phonon coupling coefficient decreases, the electron-phonon coupling increases, the Raman activity increases, and the Raman cross section increases. It is clear that as the solvent polarizability increases, the π-electron energy gap increases the modulation of the CC bond vibration.

3.4. Phase transition on the optical properties of β-Car

Investigation on β-Car solution in different phases should give more information on its electronic and vibrational states. Physical properties of carotenoids in a condensed phase were pioneered by Hashimoto et al., whose study included a wide range of researches on different types of films and their optical properties. Not surprisingly, their work provides valuable information on electronic and vibrational dynamics of optically forbidden 2^1A_g-A_g transition. Their observation of soliton-like excitations gave a new method in studying the physical properties of carotenoids and related their optical properties to the one-dimensional conducting polymers. The physical properties of β-Car in solid phase and liquid phase are quite different due to the polyene structure and the solvent effect. These two effects jointly lead to variation on optical response when the solution is in different phases.

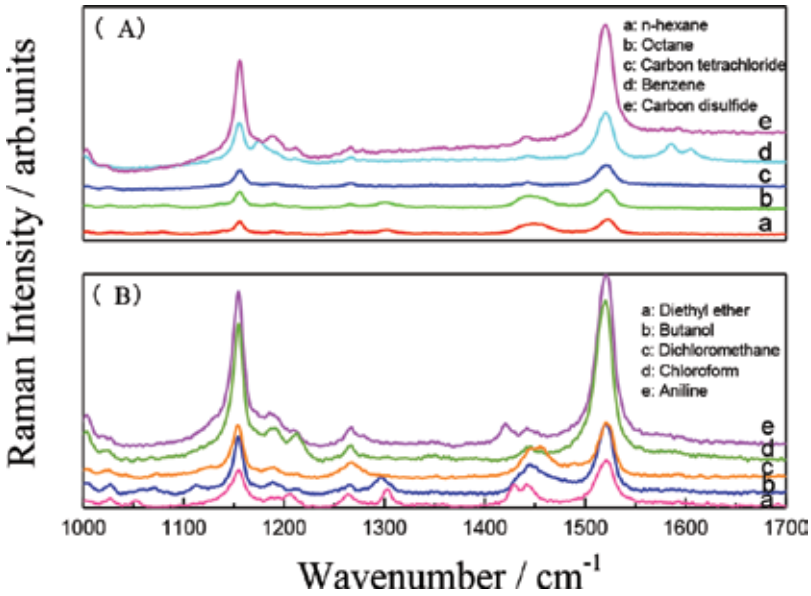

Figure 11. Raman spectra of β-Car in (a) non-polar solvents and (B) polar solvents.

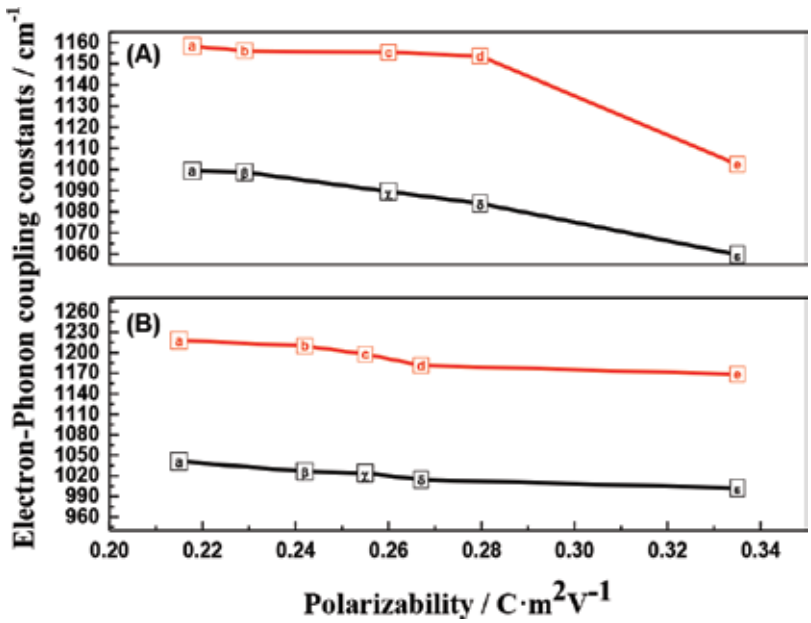

Figure 12. The relationship between electron-phonon coupling coefficient and polarizability in (a) non-polar and (b) polar solvents.

Figures 13 and **14** show the absorption and resonance Raman spectra of β-Car dissolved in cyclohexanol at 65–22°C. As the temperature decreases, the solution undergoes liquid-solid phase change at 20°C. From **Figure 13**, we can find that with a decreasing temperature, the

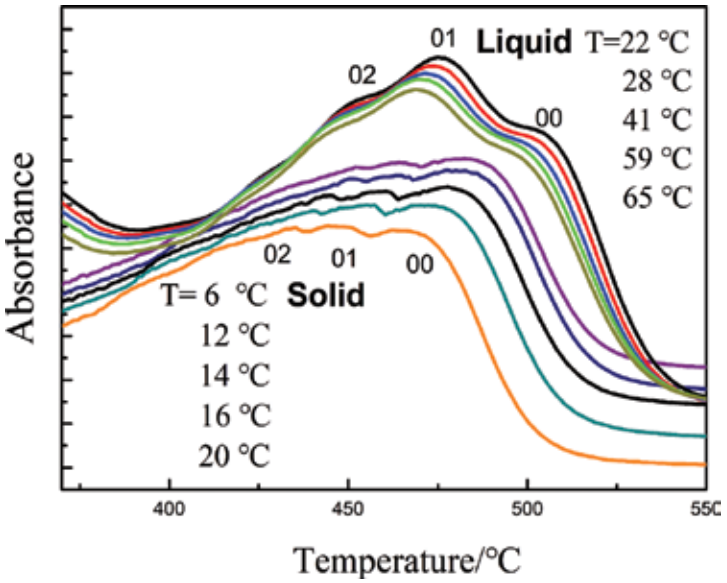

Figure 13. Absorption spectra of β-Car dissolved in cyclohexanol in different temperatures.

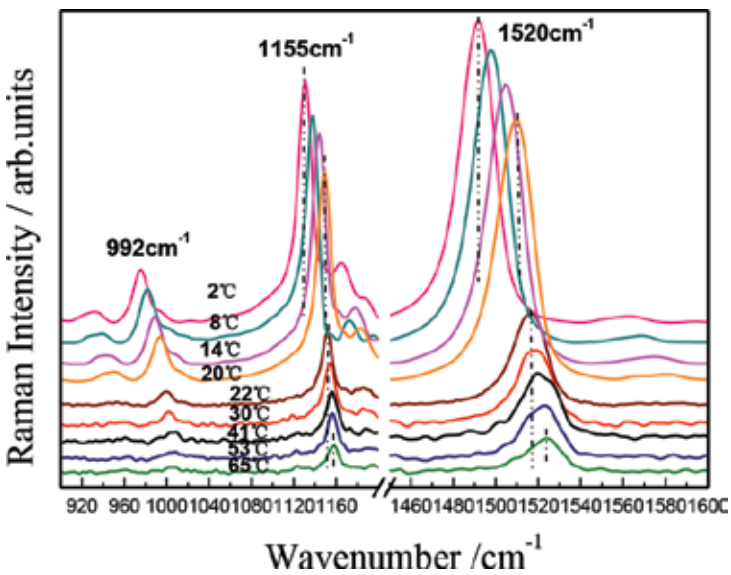

Figure 14. Raman spectra of C—C and C=C vibration of β-Car dissolved in cyclohexanol in different temperatures.

absorption spectra of β-Car molecules red-shift when the solution is in both liquid and solid phases. The solid phase (R = 2.24 nm/°C) absorption spectral red-shift is greater than that of liquid phase (R = 0.23 nm/°C). This result shows that the electron-phonon coupling of β-Car in solid phase has a more sensitive temperature dependence compared to the liquid phase. From **Figure 14**, the resonance Raman spectra show a similar changing tendency upon temperature.

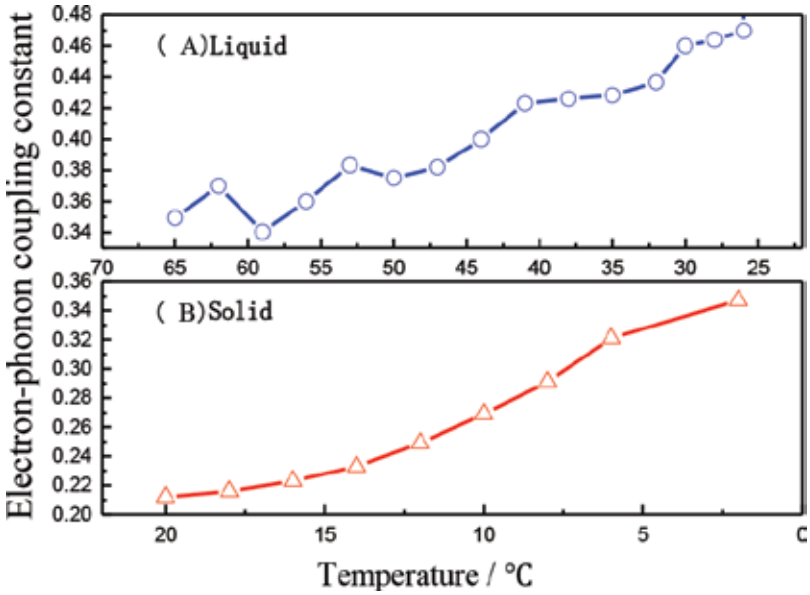

Figure 15. Temperature dependence of β-Car molecular electron-phonon coupling coefficient λ (A) before and (B) after phase transition.

With the decrease of temperature, the CC modes red-shift and their intensity enhance. The shift magnitude becomes larger after the solution phase transition, which is consistent with the absorption results. In the solid phase, there is no Brownian motion, the molecular density increases, and the movement of the β-Car molecule is prevented. Increasing the molecular structure of β-Car in an orderly manner reduces the energy of the system, the π-electron energy gap is greatly affected by temperature, the modulation of CC bond vibration is enhanced, the coupling of electron-phonon is enhanced, and the Raman-active mode red-shift is accelerated. The spectral intensity increases. According to the relationship between energy gap E_g and the coupling coefficient $E_g \sim \exp.(1/2\lambda)$, as well as λ and $\omega 1$, $\omega 2$, $2\lambda \sim (\omega^1\ \omega^2\ \omega^3)^2$, the relationship between temperature and coupling coefficient can be calculated [41]. **Figure 15** shows the relationship between the electron-phonon coupling coefficient and the temperature before and after the phase change.

4. Conclusion

Resonance Raman spectroscopy is one of the main spectral technologies for the study of linear polyene molecules. Raman spectroscopy is the result of π-electron energy gap modulation of CC atoms. External fields such as temperature, pressure, solvent effect, and phase transition have influence on the degree of ordering of polyene molecules, the effective conjugate length, and the degree of electron delocalization, and the physics behind the phenomenon of the influence by different external fields on these characteristics are not the same. Changing the system energy, the absorption spectrum would show a red-shift or a blue-shift, and the

corresponding electron-phonon coupling increases or decreases. Finally, different modulation modes are generated for the C–C and C=C vibration. In general, when the system energy decreases, the π electron energy gap is reduced. With a decreasing energy gap, the modulation is enhanced, and the electron-phonon coupling is strengthened. Raman spectra will redshift and their intensity is enhanced and vice versa.

Acknowledgements

The authors gratefully acknowledge the financial support by the National Natural Science Foundation of China (NSFC) (11574113, 1374123, and 11604024).

Conflict of interest

The authors declare no conflict of interest.

Thanks

This review dedicates to our respectable tutor Z. W. Li for his outstanding contribution and hardworking in the establishment of our laboratory.

Author details

Nan Gong[1], Zuowei Li[1], Chenglin Sun[2] and Zhiwei Men[1]*

*Address all correspondence to: zwmen@jlu.edu.cn

1 Coherent Light and Atomic and Molecular Spectroscopy Laboratory, College of Physics, Jilin University, Changchun, People's Republic of China

2 Key Laboratory of Physics and Technology for Advanced Batteries, College of Physics, Jilin University, Changchun, People's Republic of China

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Chapter 4

Microbiological Synthesis of Carotenoids: Pathways and Regulation

Pamela Córdova, Marcelo Baeza,
Víctor Cifuentes and Jennifer Alcaíno

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.78343

Abstract

Carotenoids are naturally produced by plants, algae, and some bacteria and fungi, fulfilling functions as accessory photosynthetic pigments and antioxidants. Among carotenoids, the xanthophyll astaxanthin stands out for its antioxidant and nutraceutical properties, which are beneficial to human health, and also for its use in the aquaculture industry as nutritional supplement of salmonid fish. Many studies have focused on the search of natural sources of astaxanthin as an alternative production that guarantees the beneficial properties of this compound. In nature, few astaxanthin-producing organisms are known, being the microalgae *Haematococcus pluvialis* and the yeast *Xanthophyllomyces dendrorhous* the most promising microbiological systems for the biotechnological production of this carotenoid. In this chapter, we describe the carotenogenic pathways in these microorganisms and the proposed carotenogenesis regulation mechanisms. As an example, the influence of the carbon source, the regulation by catabolic repression and by sterols in the carotenogenesis in the yeast *X. dendrorhous* is described.

Keywords: astaxanthin, *Xanthophyllomyces dendrorhous*, *ergosterol*, *catabolite repression*

1. Introduction

Currently, carotenoids are valuable molecules due to their use in different industries such as chemical, pharmaceutical, poultry, food and cosmetics. These pigments have coloring and antioxidant properties that have attracted the attention of researchers and industries as they have useful applications on several fields [1]. Carotenoids are naturally occurring lipid-soluble

pigments, the majority being C_{40} terpenoids, which act as membrane-protective antioxidants scavenging O_2 and peroxyl radicals; their antioxidant ability is apparently attributed to their structure, which in some cases (for example: astaxanthin) contain two terminal rings joined by a double conjugated chain or polyene system. Carotenoids are classified in two major groups: i) carotenes, composed only by carbon and hydrogen, and ii) xanthophylls, which are oxygenated derivatives [2]. Approximately 600 different carotenoid structures have been described to date that can be found in most life forms and have various functions ranging from their original role as photosynthetic pigments to antioxidants, precursors of vitamin A or pigments involved in the visual attraction of pollinating animals [2].

Commercial carotenoids are mainly obtained by extraction from plants and by chemical synthesis. However, some problems regarding seasonal and geographic variability that cannot be controlled arise in the case of production and marketing of several colorants of plant origin. On the other hand, the chemical synthesis may generate hazardous waste that can affect the environment. Unlike these traditional methods, the microbial production of carotenoids turns into a great opportunity of safe use. Microbial production has the advantage to use low-cost substrates, resulting in lower production costs. This explains the increasing interest in the production of microbial carotenoids as substitutes for synthetic carotenoids used as colorants in food and feed industries [1]. Among carotenoids, the xanthophyll astaxanthin stands out for its antioxidant and nutraceutical properties that are beneficial to human health, and for its use in the aquaculture industry as nutritional supplement of salmonid fish [3]. Many studies have pointed to search for a natural source of astaxanthin as an alternative production method that guarantees the beneficial properties of the mentioned compound. In nature, few astaxanthin-producing organisms are known, being the microalgae *Haematococcus pluvialis* and the yeast *Xanthophyllomyces dendrorhous* the most promising microbiological systems for the biotechnological production of this carotenoid. In this context, *X. dendrorhous* produces unmodified astaxanthin as the main carotenoid, does not require light for its growth and pigmentation and can use various carbon sources having a relatively rapid growth [2, 4]. In contrast, the production of astaxanthin by *H. pluvialis* requires specific conditions during its cultivation, because it changes its structure during the growth cycle. This means that the physical properties and nutrient requirements of the alga change during the culture process, altering the optimal conditions for growth and carotenoid production [1].

Despite the potential of *X. dendrorhous*, the specific production of astaxanthin by wild-type strains of this yeast is too low to be a commercially competitive source [5]. Due to the above, many researchers have tried to improve the production of astaxanthin using several methods as the optimization of culture conditions, classical random mutagenesis methods, genetic and metabolic engineering strategies [1, 4, 6–15]. These studies have led to an extensive knowledge about the biology of this yeast and the carotenoid synthesis pathway. However, in recent years, interest has been focused on elucidating the regulatory mechanisms operating on the production of carotenoids in this yeast. The carbon source is the most studied parameter that influences carotenogenesis in *X. dendrorhous*. Glucose and other fermentable sugars are

initially metabolized by the glycolytic pathway, followed by alcoholic fermentation, even in the presence of oxygen. On the other hand, non-fermentable carbon sources, such as ethanol and succinate, are metabolized through acetyl-CoA oxidation entering directly to the citric acid cycle. In general, non-fermentable carbon sources as ethanol, enhance the synthesis of carotenoid pigments in *X. dendrorhous* [1, 16, 17] and a relation between carotenoid production and ergosterol and fatty acid synthesis have been reported, since these pathways use the same substrates that derive from the mevalonic acid (MVA) pathway [18–20].

This chapter is focused on the microbiological synthesis of astaxanthin, taking as example the microorganisms *X. dendrorhous* and *H. pluvialis* since both are considered among the most promising for biotechnological production of this carotenoid. Also, the potential regulatory mechanisms that influence carotenogenesis is described, particularly, the influence of the carbon source and the regulation of carotenogenesis by catabolic repression in the yeast *X. dendrorhous*.

2. Importance of astaxanthin

Astaxanthin (3,3′-dihydroxy-β, β′-carotene-4,4′-dione) belongs to the xanthophyll group and its structure consists of two terminal rings joined by a polyene chain (**Figure 1**). In addition to the existence of cis or trans geometric isomers, astaxanthin presents 3 configurational isomers: two enantiomers (*3R, 3′R* and *3S, 3′S*) and one meso form (*3R, 3′S*), due to the existence of two asymmetric carbons located at the 3, 3′ positions of the β-ionone ring with hydroxyl group (–OH) on both ends of the molecule [21, 22]. The stereoisomers (3*S*, *3′S*) and (3*R*, 3′*R*) are the most abundant in nature. *H. pluvialis* biosynthesizes the (3*S*, 3′*S*)-isomer whereas the yeast *X. dendrorhous* produces the (3*R*, 3′*R*)-isomer, while synthetic astaxanthin comprises isomers (3*S*, 3′*S*), (3*R*, 3′*S*) and (3*R*, 3′*R*) [22].

Being a xanthophyll, astaxanthin possesses chemical properties and physiological characteristics of these compounds: it is highly lipophilic and shows an intense red color based on the light absorbed by its polyene system [22]. Due to its 3-hydroxyl and 4-keto functional groups in the terminal rings, astaxanthin has a higher polarity than other carotenoids and an antioxidant activity greater than that of β-carotene, lutein, zeaxanthin and canthaxanthin [5, 23, 24]. Due to its high antioxidant activity, there is an increasing number of studies referring to the astaxanthin beneficial properties for human health including several properties such as anti-inflammatory, anti-diabetic, antibacterial, immunostimulant, photoprotective, neuroprotective, anticancer and benefits to cardiovascular health, among others [2, 5, 22]. From an economical point of view, astaxanthin is the third most important carotenoid in the carotenoid global market after β-carotene and lutein. Astaxanthin market reached the 29% of total carotenoid sales with a global market size of $225 million, estimating to increase to $253 million by 2018, approximately [1]. Currently, the vast majority of the commercial offer corresponds to synthetic astaxanthin. However, the use of chemical compounds as food additives has been strictly regulated, favoring food free of them from both, the consumers and the

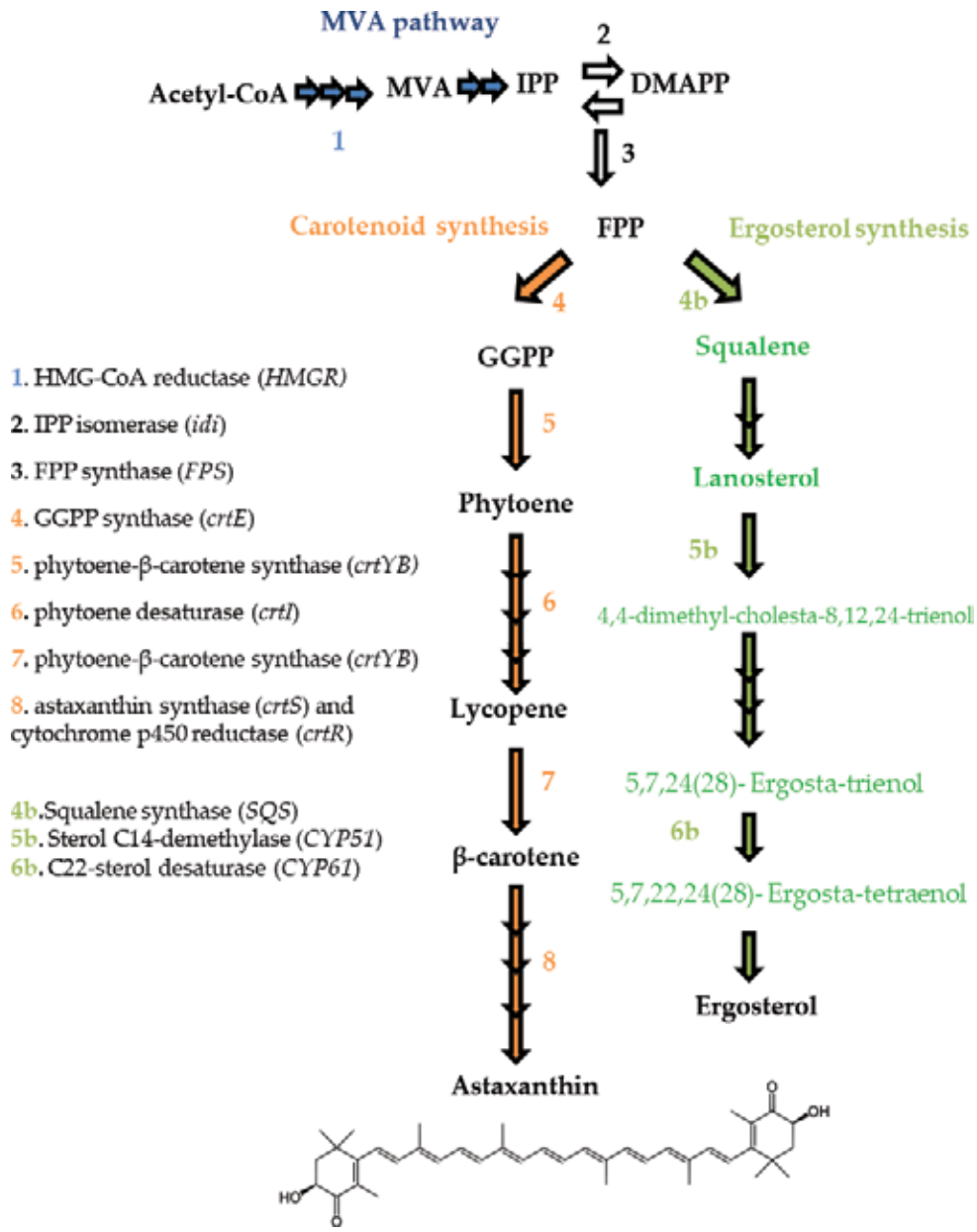

Figure 1. Overview of *X. dendrorhous* carotenogenesis and related pathways. The steps for carotenoid biosynthesis from IPP, generated via the MVA pathway, are shown. Also, an abbreviated ergosterol synthesis pathway based on the route described in *S. cerevisiae* is schematized (in green), highlighting enzymes whose genes have been functionally characterized in *X. dendrorhous (*homologous to those present in *S. cerevisiae)* [19, 66]. MVA: mevalonate; IPP: isopentenyl pyrophosphate; DMAPP: dimethylallyl pyrophosphate; FPP: farnesyl pyrophosphate; GGPP: geranylgeranyl pyrophosphate.

authorities [1, 2, 5]. In this context, the society nowadays is looking toward "green" solutions; thus, astaxanthin from natural sources, as from microorganisms, seems to be more favorable than its synthetic counterpart due to structure, function, application and security [25, 26].

http://dx.doi.org/10.5772/intechopen.78343

3. Microbiological synthesis of astaxanthin

In general, the microbiological synthesis of carotenoids has conservative steps among the different species of carotenogenic microorganisms. The most conserved carotenoid biosynthesis pathways in eukaryotic microorganisms involve three general steps [1]:

1. The synthesis begins with the conversion of acetyl-CoA to 3-hydroxy-3-methyl glutaryl-CoA (HMG-CoA), catalyzed by HMG-CoA synthase. Then, HMG-CoA is converted into mevalonic acid (MVA), being the first precursor of the terpenoid biosynthetic pathway. The following steps include two sequential phosphorylation reactions performed by the enzymes mevalonate kinase and phosphomevalonate kinase, respectively, and a final decarboxylation step catalyzed by phosphomevalonate decarboxylase to produce IPP [1, 20].
2. IPP is isomerized to dimethylallyl pyrophosphate (DMAPP). The addition of three IPP molecules to DMAPP, catalyzed by prenyltransferases, produce geranylgeranyl pyrophosphate (GGPP). Then, the condensation of two molecules of GGPP produces phytoene (the first C_{40} carotene of the pathway), which is subsequently desaturated to produce lycopene [1].
3. Lycopene undergoes many modification reactions and several cyclic carotenoids derived from lycopene such as β-carotene, γ-carotene, torulene, torularhodin and astaxanthin [1].

Despite the common steps in carotenogenesis, there are particular differences in the synthesis pathways between different microorganisms. In the following sections, the carotenogenic pathways in the microalgae *H. pluvialis* and in the yeast *X. dendrorhous* are described.

3.1. Astaxanthin biosynthesis in *X. dendrorhous*

It is currently believed that the main function of carotenoids in *X. dendrorhous* is to protect the yeast against damage caused by oxidative stress. This hypothesis is supported by the fact that strains that do not produce astaxanthin are more sensitive and grow less in the presence of ROS [23]. In addition, it has been reported that the presence of certain oxygen species increases the total carotenoid content [23, 24]. Other evidence points to the fact that *X. dendrorhous* has significantly lower catalase activity than other yeasts such as *Saccharomyces cerevisiae*, so carotenoids could compensate this low activity to help to preserve the viability of continuously growing yeasts [23]. On the other hand, *X. dendrorhous* does not have a cytosolic version of a superoxide dismutase, and carotenoids could also be compensating the lack of this enzyme [23]. *X. dendrorhous* is the only known yeast that produces astaxanthin *de novo* [22] and it has been approved by the FDA (Food and Drug Administration) for the commercial production of astaxanthin; therefore, it is a good candidate to allow the natural production of astaxanthin [27]. Knowledge about the biology of this yeast has increased and several methodologies to manipulate it have been developed, but still several aspects need to be improved for an economically competitive production of astaxanthin from *X. dendrorhous* [27].

The synthesis pathway preserves the basic steps of carotenoid synthesis in this yeast; however, some steps involve characteristic enzymes in fungi and other characteristic enzymes in

X. dendrorhous [28–30]. Many studies have attempted to improve astaxanthin production in *X. dendrorhous*, contributing to our current knowledge of the genetic control of carotenogenesis in this yeast (**Figure 1**). As in other eukaryotes, the synthesis of carotenoids in *X. dendrorhous* derives from the MVA pathway. MVA is formed by the condensation of three molecules of acetyl-CoA [31], followed by two phosphorylation reactions by two different kinases and one decarboxylation, giving rise to isopentenyl pyrophosphate (IPP, C_5), which is the building block of all isoprenoids [5, 20, 31]. IPP is isomerized to dimethylallyl pyrophosphate (DMAPP) by the IPP isomerase, encoded by the *idi* gene [32]. In the next steps, a molecule of DMAPP is sequentially condensed with three molecules of IPP to generate geranylgeranyl pyrophosphate (GGPP, C_{20}); these steps involve the prenyl transferase enzymes farnesyl pyrophosphate synthase and geranylgeranyl pyrophosphate synthase, encoded by the *FPS* and *crtE* genes, respectively [33]. Subsequently, by means of a bifunctional enzyme phytoene-β-carotene synthase (PBS) with two activities (lycopene cyclase and phytoene synthase), that in fungi are encoded by the same gene (*crtYB* in the case of *X. dendrorhous*), two molecules of GGPP are condensed giving rise to phytoene (C_{40}), the first carotenoid of the pathway, which is colorless [28]. Then, phytoene undergoes four desaturation reactions catalyzed by a single enzyme phytoene desaturase (PDS, product of the *crtI* gene) forming lycopene (a red pigment) [5, 34]. The latter is converted to β-carotene by the lycopene cyclase activity of the bifunctional PBS enzyme. Finally, β-carotene is oxidized by the incorporation of a hydroxyl group in position 3 and a keto group in position 4 of both β-ionone rings of β-carotene generating astaxanthin as the final product. Unlike other organisms that produce astaxanthin, in *X. dendrorhous* a single enzyme catalyzes these last oxidizing steps from β-carotene to astaxanthin named astaxanthin synthase (CrtS, encoded by the *crtS* gene), which is a cytochrome P450 monooxygenase [29]. Astaxanthin synthase requires a redox partner, a cytochrome P450 reductase encoded by the *crtR* gene in *X. dendrorhous* [30, 35, 36], which provides the necessary electrons for the enzyme catalysis. The main pigments produced by *X. dendrorhous* are xanthophylls, of which astaxanthin represents 83–87% of total carotenoids [5].

3.2. Biosynthesis of astaxanthin in the microalgae *H. pluvialis*

Several algae and microalgae are able to produce astaxanthin, but *H. pluvialis* is the dominant specie for commercial astaxanthin [22]. *H. pluvialis* contains 1.5–3% of (3S, 3′S)-astaxanthin by dry weight, mainly as monoesters [37]. The isomer (3S, 3′S)-astaxanthin is the preferred form for human applications, for this reason, *H. pluvialis* is an attractive natural source of this pigment [5].

For the production of pigments, the microalgae *H. pluvialis* is usually grown in a two-stage batch process [5]. The first stage (green stage) is necessary to obtain enough biomass for an efficient carotenoid production. In this stage, microalgae are grown in presence of sufficient nutrient supply, optimum pH and temperature, and low irradiation. Since, the astaxanthin synthesis is induced under stress conditions, the second stage of growth (the reddening stage), consists in exposing the cells to stress conditions such as high light irradiation (sun light), nutrient deprivation (mainly nitrogen and phosphate deprivation), and high temperature and/or high salt concentration [26].

As in *X. dendrorhous*, the biosynthesis of astaxanthin in *H. pluvialis* also begins with the synthesis of IPP, which is a key intermediate of carotenoid synthesis. In general, and depending

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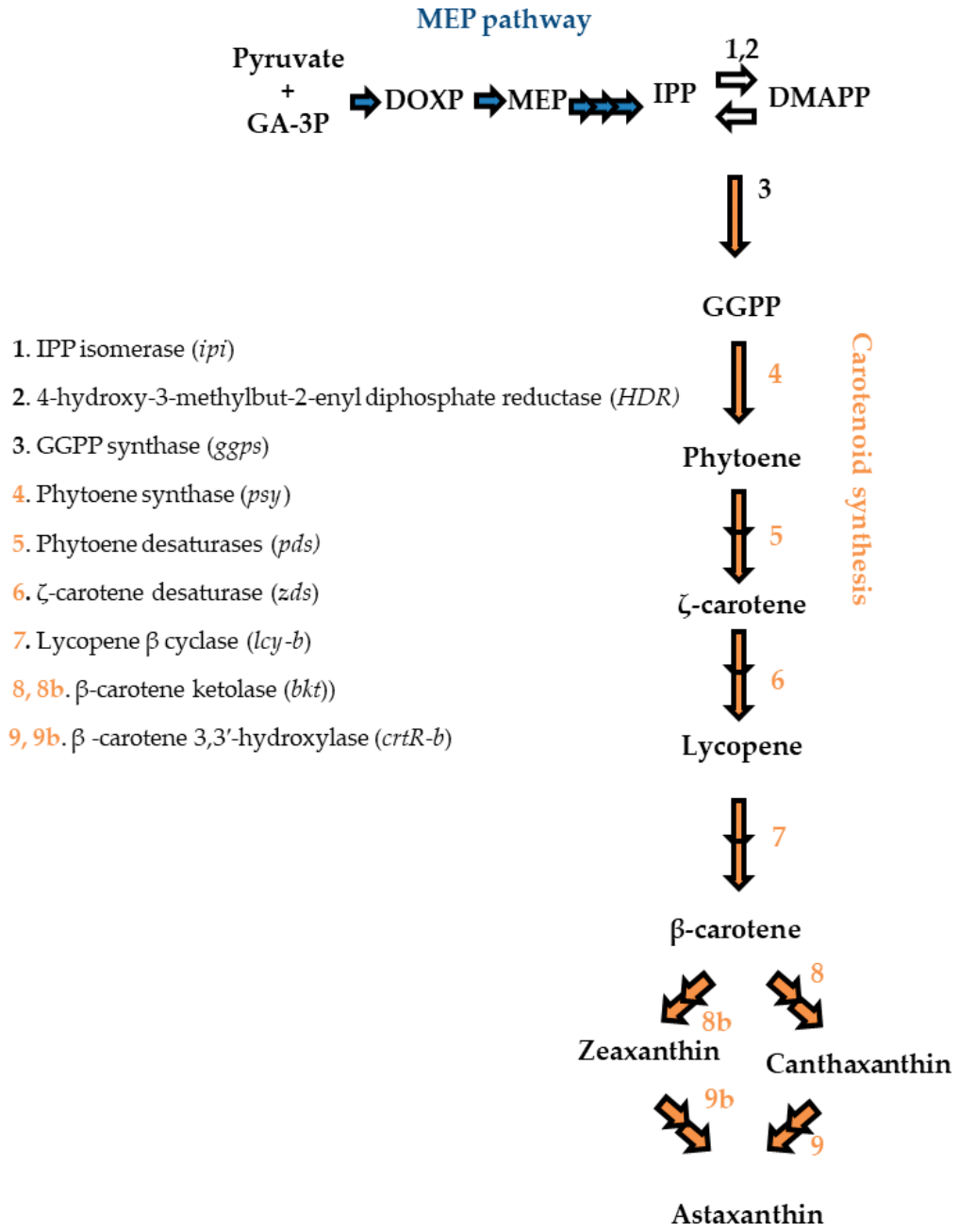

Figure 2. Overview of *H. pluvialis* carotenogenesis. The steps for astaxanthin biosynthesis from IPP, generated via the MEP pathway, in *H. pluvialis* are shown. Enzymes involved in each step are listed at left and the corresponding genes are indicated in parenthesis. GA-3P: glyceraldehyde 3-phosphate; DOXP: 1-deoxy-D-xylulose-5-phosphate; MEP: methylerythritol 4-phosphate; IPP: isopentenyl pyrophosphate; DMAPP: dimethylallyl pyrophosphate; GGPP: geranylgeranyl pyrophosphate.

on the organism, IPP may be produced by two different pathways, the i) MVA pathway (cytosolic) and a ii) pathway located in the chloroplast known as the MEP (Methylerythritol 4-phosphate) or as the DOXP (due to the formation of 1-deoxy-D-xylulose-5-phosphate in the

first stage of the pathway) pathway [38–40]. Previous studies have shown that in *H. pluvialis*, the intermediate IPP most probably derives from the MEP pathway as it lacks three key enzymes of the mevalonate pathway involved in the formation of IPP from acetoacetyl-CoA [41]. To date, the enzymes required for the conversion of photosynthesis derived products i.e., pyruvate and glyceraldehyde-3-phosphate into isopentenyl pyrophosphate through the DOXP pathway inside *H. pluvialis* chloroplasts has been extensively studied [41], being this, the most likely source of IPP in *H. pluvialis* cells.

The carotenogenic pathway described for *H. pluvialis* is presented in **Figure 2**. As mentioned before, the astaxanthin synthesis precursor IPP derives from the DOXP (or MEP) pathway. As in *X dendrorhous*, the first step is the isomerization of IPP to DMAPP. It has been long assumed that this conversion was catalyzed exclusively by isopentenyl pyrophosphate isomerase (IPI, encoded by *ipi* genes in *H. pluvialis*) [39, 42]. However, recent transcriptomic studies suggest that neither of the two *ipi* genes of *H. pluvialis* (*ipi1* and *ipi2*) are upregulated during cellular accumulation of astaxanthin [41]. On the contrary, suggestions have been made that another enzyme of similar activity, 4-hydroxy-3-methylbut-2-enyl diphosphate reductase (HDR) is more likely to be responsible for catalyzing the interconversion between IPP and DMAPP [41]. However, the contribution of these three enzymes to this step of astaxanthin biosynthesis is still unclear. As in *X. dendrorhous*, the isoprenoid chain is elongated by the addition of a molecule of DMAPP and subsequent additions of three molecules of IPP, being these steps catalyzed by the geranylgeranyl pyrophosphate synthase (GGPS) enzyme giving rise to GGPP [43]. The first committed step of carotenoid synthesis is the formation of phytoene from two molecules of GGPP which are condensed in a head-to-tail manner by the enzyme phytoene synthase (PSY) [43]. It must be noted that the same step in *X. dendrorhous* is catalyzed by the bifunctional enzyme PBS [28]. Then, phytoene is desaturated four times. In *H. pluvialis*, these steps involve two phytoene desaturases (PDS) and a ζ-carotene desaturase (ZDS), and two plastid terminal oxidases (PTOX1, PTOX2) acting as co-factors [44, 45], giving as final product the red colored carotene lycopene [43]. These steps constitute other difference with the synthesis of carotenoids in *X. dendrorhous*, where a single enzyme catalyzes the four desaturations necessary for the synthesis of lycopene from phytoene [34]. Both termini of lycopene are cyclized by lycopene cyclases (LCY-e and LCY-b). In most organisms, cyclization of an extreme of lycopene results in the production of α-carotene (precursor of lutein) and β-carotene (precursor of astaxanthin, among others). In *H. pluvialis,* the carbon flux is directed mainly through the production of β-carotene [41]. The final oxygenation steps that lead to astaxanthin from β-carotene are catalyzed by two different enzymes: a β-carotene ketolase (BKT) and a β-carotene hydroxylase (CrtR-b) [46–48]. This is another difference with the astaxanthin synthesis in *X. dendrorhous*, in which the astaxanthin synthase yields the hydroxylation and ketolation of the β-carotene β-ionone rings [29].

4. Potential mechanisms that regulate the synthesis of astaxanthin

The astaxanthin synthesis pathway has been extensively studied, and most of genes and enzymes involved are currently known. More recent studies have focused on elucidating the possible regulation mechanisms of carotenogenesis. In the case of yeast *X. dendrorhous*, special

emphasis has been placed on understanding the effect of the carbon source on carotenogenesis and how this pathway is affected by other related pathways, such as the synthesis of sterols. In the case of *H. pluvialis*, special interest has been placed on the effect of small molecules on the synthesis of astaxanthin.

4.1. Regulation of carotenogenesis in *X. dendrorhous*

An important function of astaxanthin in *X. dendrorhous* is the inactivation of singlet and oxygen radicals, which is consistent with the fact that astaxanthin production increases in the presence of these reactive oxygen species [5, 23]. In addition, it has been observed that high light intensity inhibits the growth of the yeast and the content of carotenoids. However, at low light intensities, light has a positive regulatory effect on the synthesis of carotenoids [5].

It is known that *X. dendrorhous* is able to grow using various carbon sources, among them: glucose, sucrose, maltose, xylose, starch, succinate, glycerol and ethanol. Several studies have shown that there is a relationship between the carbon source used by the yeast and the synthesis of carotenoids. This effect is observed in both: in the amount of total pigments and in their composition [16, 17].

As in other yeasts, *X. dendrorhous* is capable of carrying out two types of metabolisms depending on the carbon source that is present in the culture medium: i) a fermentative and ii) an aerobic metabolism. In previous studies, it has been shown that astaxanthin production decreases during fermentative metabolism (in presence of fermentable carbon sources as glucose or fructose) and it increases during aerobic metabolism (with non-fermentable carbon sources as succinate or ethanol) [16, 17, 49]. Also, it has been observed that carotenoid content is significantly higher when *X. dendrorhous* is cultivated in complete medium (YM) supplemented with different non-fermentable carbon sources (xylose, succinate, sodium acetate, glycerol and ethanol), compared with the carotenoid content when the yeast is cultured in presence of glucose [17]. In cultures supplemented with glucose as the sole carbon source, carotenogenesis is induced only after the culture medium runs out of glucose. While in cultures using succinate as the sole carbon source, it was observed that the production of carotenoids coincides with the growth of the yeast, increasing steadily until reaching the stationary phase of growth. This shows that the production of carotenoids starts earlier and it is higher when a non-fermentable carbon source is used in cultures [17].

On the other hand, studies of carotenogenic gene transcripts (*crtI*, *crtYB* and *crtS*), show that their levels reach their maximum value at the late exponential phase of growth coinciding with the induction of carotenogenesis, the exhaustion of glucose in the medium and with the beginning of the consumption of ethanol produced as result of sugar fermentation [17, 49]. It has also been observed that the addition of glucose to the culture medium decreases the transcript levels of genes *crtYB*, *crtI* and *crtS*, which correlates with a complete inhibition of pigment synthesis. On the other hand, the addition of ethanol to the culture medium of the yeast causes an induction of the expression of the *crtYB* and *crtS* genes, and promotes the synthesis of carotenoids [16]. Furthermore, the promoter region of the *crtS* gene contains four potential CreA binding motifs [50], which is a negative regulator involved in glucose repression in *Aspergillus nidulans* [51]. According to this background, it is clear that glucose causes suppression of carotenogenesis in *X. dendrorhous.*

It is well known that glucose has a global effect on cellular metabolism; generally, when this sugar is present in the culture medium, the expression of genes involved in the metabolism of alternative to glucose carbon sources and secondary metabolism is repressed [52]. This phenomenon is known as "catabolic repression" or "repression by glucose" [53] and could be responsible for the repression of carotenogenic genes in *X. dendrorhous* during fermentative metabolism.

4.1.1. Catabolic repression: mechanism and components

In microorganisms of free life, the availability of nutrients is in constant change and is the main factor regulating their growth and development. For yeasts, as for many other microorganisms, glucose is the preferred source of carbon and energy. Therefore, it is not surprising that glucose, in addition to its function as a nutrient, plays an important regulatory role in the metabolism of microorganisms. Thus, a high concentration of glucose in the medium resembles optimal growth conditions to the cellular machinery, causes the induction of various signal transduction pathways, and the activation or inactivation of various proteins. The regulatory role of glucose is more prominent at the transcription level and the general mechanism of catabolic repression involves a parallel decrease in the transcript levels of the target genes and, consequently, of the proteins they encode [52, 54].

Catabolic repression has been widely studied in *S. cerevisiae* and in general, genes that are under regulation by glucose repression encode enzymes that are involved in gluconeogenesis, Krebs cycle, glyoxylate cycle, respiration, mitochondrial development, uptake and metabolization of carbon sources alternative to glucose (such as the genes *GAL*, *SUC* and *MAL*) and high affinity glucose transporters [54, 55]. On the other hand, genes that encode a variety of transcriptional activators are also repressed by glucose. Finally, a large group of genes encoding proteins that are involved in the response to various types of stress highlights, as they have STRE elements (stress response element) in their promoter regions, which are also repressed in the presence of glucose [54].

One of the ways in which glucose influences gene expression is by facilitating the action of negative regulators [52], among them, the Mig1 factor (homologous to CreA in *A. nidulans*) is a DNA binding protein that recognizes and binds to specific sequences called "Mig1 boxes" (with the consensus sequence (G/C)(C/T)GGGG)) in the promoters of the target genes. Several studies have identified genes potentially encoding Mig1 in yeasts like *Kluyveromyces lactis*, *Kluyveromyces marxianus*, *Schizosaccharomyces pombe*, *Candida albicans* [52] and recently in *X. dendrorhous* [56].

In general, glucose repression in *S. cerevisiae* (**Figure 3**) mainly depends on the subcellular localization of the Mig1 regulator. At high glucose levels, the repressor Mig1 is dephosphorylated and localizes at the nucleus where it recognizes and binds to "Mig1 boxes" in the promoter region of the target genes. Then, Mig1 recruits a co-repressor complex formed by the Cyc8 and Tup1 proteins that represses the transcription of the target genes. In the absence of glucose, Mig1 is phosphorylated by the Snf1 kinase complex, loses its interaction with the Cyc8–Tup1 complex, and it is exported to the cytoplasm [57–59]. In *S. cerevisiae*,

http://dx.doi.org/10.5772/intechopen.78343

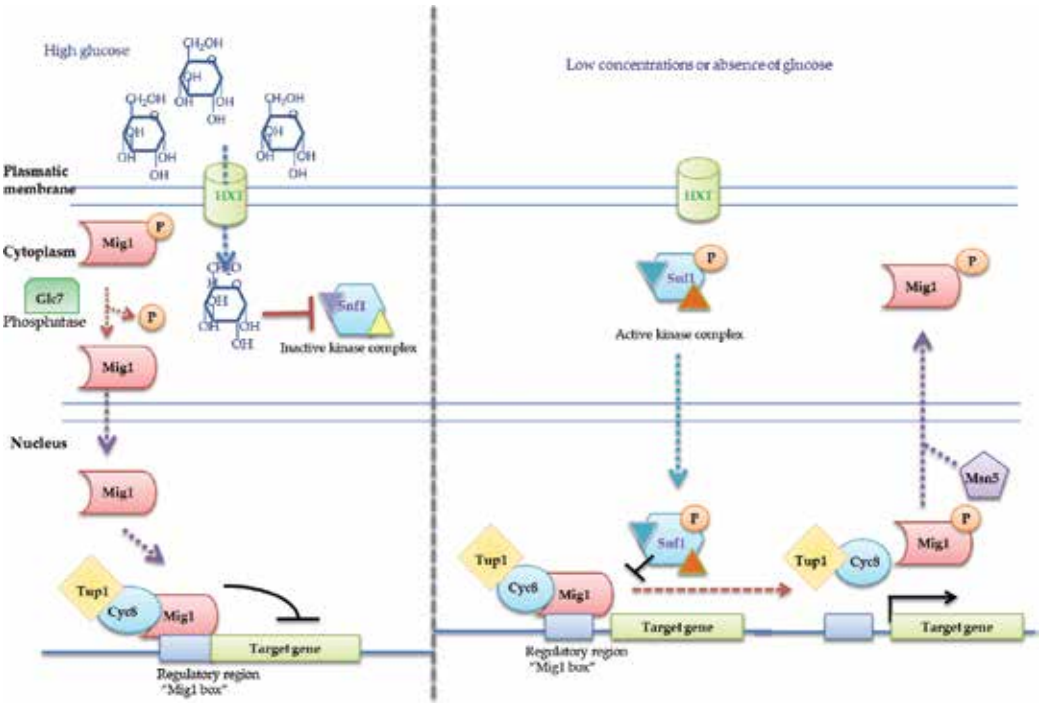

Figure 3. Catabolite repression mechanism in yeasts. Scheme of the principal components of the general catabolite repression mechanism. At high levels of glucose, Mig1 is dephosphorylated by phosphatase Glc7 and enters the nucleus where it recognizes regulatory sequences in the target gene promoters. Then, Mig1 recruits a co-repressor complex formed by Cyc8 and Tup1 that represses the expression of the target genes at transcriptional level. In absence of glucose, Mig1 is phosphorylated by the kinase complex Snf1, and loses its interaction with Cyc8-Tup1 complex, being exported to the cytoplasm [52].

the co-repressor complex Cyc8–Tup1 is considered as a global transcriptional co-repressor, because it regulates the expression of more than 180 genes, including those regulated by glucose. The proteins Cyc8 and Tup1 belong to evolutionarily highly conserved protein families, and similar repressors have been described in yeasts, worms, flies and mammals [60]. In *S. cerevisiae*, the *CYC8* and/or *TUP1* genes knock-out mutations are not lethal, but have pleiotropic effects causing slow growth, flocculation, sporulation and loss of certain aspects of glucose repression, among others [52, 60, 61].

4.1.2. Catabolic repression and carotenogenesis regulation in X. dendrorhous

In view of the diverse background that suggests that carotenogenesis is regulated by catabolic repression, recent studies have advanced characterizing the catabolic repression mechanism in *X. dendrorhous*, including its components and role in the regulation of carotenogenesis. Several evidences suggest a functional catabolic repression mechanism in *X. dendrorhous* [16, 56, 59]. For example, extracellular α-glucosidase and invertase activities were not detected in *X. dendrorhous* cultures when glucose was used as a carbon source, suggesting

catabolic repression of these enzymes [62, 63]. This indicates that this regulatory mechanism is operative in *X. dendrorhous*, as genes encoding glycosyl hydrolases are well known targets of catabolic repression.

Moreover, according to descriptions of other yeasts, one consequence of catabolic repression is the preferred use of glucose over other alternative carbon sources, deferring their use until glucose has been completely consumed. The preferential use of glucose over an alternative carbon source can be evidenced by a characteristic growth rate change of the microbial culture, known as diauxic growth [59, 64]. This aspect has been evaluated in *X. dendrorhous* through experiments in which the yeast was grown in the presence of glucose and a non-preferred carbon source such as glycerol or sucrose. In both cases, a diauxic-type growth curve was obtained when the yeast was cultivated with both carbon sources simultaneously, indicating the change in the used carbon source and supporting a functional catabolic repression mechanism in *X. dendrorhous* [56, 59]. On the other hand, genes encoding the principal components of the catabolic repression mechanism has been identified and characterized in *X. dendrorhous*. Among them, a gene (*MIG1*) encoding the Mig1 transcriptional factor was identified [56]. The functionality of this gene was assessed by heterologous complementation in *S. cerevisiae*, showing that the protein encoded by the *MIG1* gene is functional and capable of mediating glucose repression [56]. Also, the function of Mig1 in *X. dendrorhous* was determined by evaluating the effect of a *mig1*$^-$ mutation on carotenoid production, gene expression and extracellular invertase activity (a known glucose repression target). To evaluate whether carotenoid production is affected in the *mig1*$^-$ mutant strain, samples were taken from cultures of the wild-type and mutant strains grown in presence of glucose at 5 different time-points representatives of different phases of growth. It was observed that the carotenoid content was higher in the *mig1*$^-$ mutant strain during almost all phases evaluated and the total carotenoid content at the final phase of growth that was evaluated (stationary phase of growth), was approximately 20% higher in the mutant strain compared to the wild-type [56]. The higher carotenoid production in the *X. dendrorhous mig1*$^-$ mutant strain strongly suggests a role of Mig1 in the regulation of carotenogenesis in this yeast. Also, in a complementary approach it was demonstrated that when glucose was added to a culture that was previously deprived of this sugar, the carotenoid synthesis stopped in the wild-type strain showing no carotenoid synthesis until 24 h later, while in the case of the *mig1*$^-$ mutant strain, carotenogenesis did not stop, suggesting that the *mig1*$^-$ mutation alleviate the glucose mediated repression of carotenogenesis [56]. Also, Marcoleta et al. showed that the addition of glucose decreased the transcript levels of the carotenogenic genes in the wild-type strain [16]. Meanwhile, the *mig1*$^-$ mutation reverts this repression at the transcriptional level [56]. Additionally, by bioinformatic analysis, possible "Mig1 boxes" were identified in the promoter regions of the carotenogenic genes *crtI*, *crtYB* and *crtS*, precisely those in which a repressing effect of glucose at the transcriptional level was also observed [16]. To confirm whether the *X. dendrorhous MIG1* gene product binds to DNA containing Mig1 boxes, Electrophoretic Mobility Shift Assays (EMSAs) were performed using biotin-labeled DNA fragments of the promoter regions of the *crtI*, *crtYB* and *crtS* containing the potential Mig1 boxes. The results indicated that the Mig1 factor of *X. dendrorhous* is capable of binding specifically to the "Mig1 boxes" present in the

analyzed promoter regions [56]. To further address the functional consequences of the *mig1*⁻ mutation in *X. dendrorhous*, extracellular invertase activity was evaluated in the wild-type and mutant strains. Invertase (encoded by the *INV* gene) catalyzes the utilization of sucrose as carbon source and it is under catabolic repression in presence of glucose [52]. When invertase activity was determined in the wild-type and *mig1*⁻ mutant strain of *X. dendrorhous* cultured in the presence of glucose, it was observed that the *mig1*⁻ mutation caused derepression of the *INV* gene which was evidenced by a higher invertase activity in the mutant strain compared to the wild-type under these conditions [56].

As mentioned before, the Mig1 factor recruits a co-repressor complex formed by the Cyc8 and Tup1 proteins to perform repression at transcriptional level. Thus, in other study, the role of these co-repressors in catabolic repression and carotenogenesis was assessed in *X. dendrorhous* [59]. The *CYC8* and *TUP1* genes were identified in *X. dendrorhous* and similar analyses to those described for *MIG1*, were performed. These analyses showed that the *cyc8*⁻ and *tup1*⁻ mutations increased the specific carotenoid production, reaching production levels at the stationary phase of growth that were approximately 90 and 40% higher in the mutant strains *cyc8*⁻ and *tup1*⁻, respectively, compared to the wild-type when glucose was used as the sole carbon source [59]. Also, the participation of these genes (*CYC8* and *TUP1*) in the repression of invertase activity was demonstrated, showing that *cyc8*⁻ and *tup1*⁻ mutations led to a derepression of the *INV* gene and to a higher invertase activity in presence of glucose, compared to the wild-type strain [59]. Furthermore, it was demonstrated that similarly to the *mig1*⁻ mutation, the *cyc8*⁻ and *tup1*⁻ mutations alleviated the glucose mediated repression of carotenogenesis, since these mutant strains continuously produced carotenoids, even when glucose was added to the culture medium [59].

So, many studies support the hypothesis that carotenogenesis in *X. dendrorhous* is regulated by the catabolic repression mechanism.

4.1.3. Relation between carotenogenesis and sterol biosynthesis

Ergosterol is the main sterol in fungi, fulfilling similar roles as cholesterol in mammalian cells. Since the synthesis of ergosterol and of fatty acids derive from the same precursors as the synthesis of astaxanthin in *X. dendrorhous*, some studies have focused in the interaction between these related pathways. In Ref. [65] was reported that an astaxanthin overproducing strain obtained by random mutagenesis had a decreased production of ergosterol and of fatty acids, which could lead to precursor accumulation favoring the astaxanthin biosynthesis. Moreover, it was also observed higher transcript levels of carotenogenic genes (*crtI*, *crtYB* and *crtS*) in this strain [65]. In the same line, it was shown that the disruption of the C22-sterol desaturase gene (*CYP61*), involved in one of the last steps of ergosterol synthesis, enhanced carotenoid production in *X. dendrorhous* [19].

In *Cryptococcus neoformans* and mammalian cells, the expression of the 3-hydroxy-3-methylglutaryl-CoA reductase (HMG-CoA reductase) encoding gene (*HMGR* gene) that is involved in the synthesis of IPP trough the MVA pathway, is regulated by sterol levels [19]. In accordance,

the transcript level of the *HMGR* gene in the *X. dendrorhous cyp61*⁻ mutant strains was higher than in the wild-type strains. This could explain, at least in part, the increased carotenoid content in these mutants, since the synthesis of carotenoid precursors through the MVA pathway could be favored in these strains, showing an interaction between both biosynthetic pathways and a potential role of sterols in the regulation of carotenogenesis in *X. dendrorhous* [19].

Similar to these results, other studies have demonstrated an increased astaxanthin production in *Phaffia rhodozyma* (anamorphic state of *X. dendrorhous*) when ergosterol levels were reduced by fluconazole treatment [65]. In a similar way, it has been described in *X. dendrorhous* that the mutation of the *CYP51* gene that encodes a cytochrome P450 monooxygenase that catalyzes the C14 demethylation of lanosterol during ergosterol biosynthesis, resulted in a reduced ergosterol production together with an increased carotenoid production compared to the wild-type strain [66]. Moreover, as for the *cyp61*⁻ mutation, the *cyp51*⁻ mutation in *X. dendrorhous* increased the *HMGR* transcript levels. A possible explanation for the increased carotenoid content in the *cyp61*⁻ and *cyp51*⁻ mutants could be the greater availability of carotenoid precursors when sterol biosynthesis is affected.

All together these results suggest that in *X. dendrorhous*, sterol levels, possible by a negative feedback mechanism, regulate at least the *HMGR* gene expression and in this way; it contributes to the regulation of carotenoid biosynthesis [19, 66].

4.2. Regulation of carotenogenesis in *H. pluvialis*

It has been extensively accepted that carotenoid synthesis in *H. pluvialis* is induced under stress conditions such as high light, salinity or carbon to nitrogen ratio [48, 67]. Regulation of the carotenogenic pathway in this microalga can be affected by numerous small molecules like plant hormones or similar compounds. In this context, among the hormones associated with stress response mechanisms and induction of astaxanthin synthesis in *H. pluvialis* are abscisic acid (ABA), jasmonic acid (JA), methyl jasmonate (MJ) or growth regulators like gibberellic acid (GA3), salicylic acid (SA) or brassinosteroid-2,4-epibrassinolide (EBR) [26]. It has been shown that all of these compounds affect the expression of numerous genes involved in astaxanthin synthesis, resulting in an up-regulation from 6- to 10-fold. Among them, SA showed the best results enhancing the astaxanthin production [67].

Studies at the mRNA levels of the carotenogenic genes: *ipi, psy, pds, crtO* and *crtR-b,* encoding the key enzymes of astaxanthin synthesis pathway, and its correlation with algal growth and astaxanthin production, suggested complex and multiple regulatory mechanisms that act at the transcriptional, translational and post-translational levels to regulate carotenogenesis in *H. pluvialis* [44]. Small molecules can exert different and multiple effects on several genes involved in the synthesis of astaxanthin. For example, when *H. pluvialis* was submitted to several nutrient stress conditions, it was observed that expression of carotenogenic genes encoding PSY, PDS, LCY, BKT and CrtR-b enzymes, were up-regulated under all the stress conditions studied. However, the extent of the transcript levels of carotenogenic genes varied among the stress conditions. Some of these genes, as *bkt* and *crtR-b*, were induced only transiently in some conditions. Moreover, studies using various inhibitors indicated that general carotenogenesis genes were regulated at transcriptional and translational levels. The induction of the general carotenoid synthesis genes showed to be independent of cytoplasmic

protein synthesis while *bkt* gene expression was dependent on *de novo* protein synthesis [48]. It is still necessary to elucidate the different mechanisms of response to these molecules and therefore understand carotenogenic gene regulation in *H. pluvialis* and potentially enhance its capacity as a commercial astaxanthin producer.

5. Conclusions

X. dendrorhous and *H. pluvialis* are the most promising natural sources for the biological production of astaxanthin, which is used in several industrial applications. Almost all the genes and enzymes involved in the carotenogenesis pathways in both microorganisms are known. Currently, efforts have been directed in order to elucidate the regulatory mechanisms acting on carotenogenesis in these microorganisms. Studies show that multiple and complex carotenogenesis regulatory mechanisms are involved acting at transcriptional, post-transcriptional and translational level, and that they could be different in these microorganisms. Regarding *X. dendrorhous* carotenogenesis, there is evidence that suggest that it is regulated by catabolic repression and by sterols levels, while in *H. pluvialis*, carotenogenesis is induced under stress conditions and it is affected by numerous small molecules like plant hormones or their analogs.

Acknowledgements

FONDECYT 1160202 and FONDECYT 1180520.

Conflict of interest

Authors declare that they have no conflicts of interest.

Author details

Pamela Córdova, Marcelo Baeza, Víctor Cifuentes and Jennifer Alcaíno*

*Address all correspondence to: jalcainog@uchile.cl

Universidad de Chile, Santiago, Chile

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Chapter 5

Biosynthesis of Carotenoids and Apocarotenoids by Microorganisms and Their Industrial Potential

Congqiang Zhang

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.79061

Abstract

Carotenoids are a large group of natural pigments, ranging from red, to orange, to yellow colors. Synthesized by plants and some microorganisms (e.g., microalgae, fungi and bacteria), carotenoids have important physiological functions (e.g., light harvesting). Apocarotenoids are carotenoid-derived compounds and play important roles in various biological activities (e.g., plant hormones). Many carotenoids and apocarotenoids have high economic value in feed, food, supplements, cosmetics and pharmaceutical industries. Despite high commercial values, they are severely undersupplied because of low abundance in natural hosts (usually a few milligrams per kilogram of raw materials). Furthermore, plants or microbes usually produce mixtures of these molecules with very similar physical and chemical properties (such as α- and β-carotenes). All these features render the extraction from natural hosts rather difficult and also very costly both from process economics and sustainable land-use viewpoints. Chemical synthesis is also expensive due to structural complexity (e.g., astaxanthin has many unsaturated bonds and two chiral regions). Biotechnology via the rapidly advancing metabolic engineering and synthetic biology approaches has led to alternative ways to attain several carotenoids and apocarotenoids at relatively high titers and yields using fast-growing microorganisms. This chapter briefly reviews the biosynthesis of carotenoids and apocarotenoids by microorganisms and their industrial potential.

Keywords: metabolic engineering, fermentation, carotenoids, astaxanthin, lycopene, carotene, retinol and ionone

1. Introduction

Carotenoids are natural red, orange or yellow pigments widely distributed in nature. The vivid color of carotenoids contributes to the beauty of many flowers, fruits and animals. For

example, the loveliness of yellow marigolds comes mainly from lutein, a yellow carotene; the redness of watermelons and tomatoes is because they are rich in lycopene, a red carotene; and the scarlet plumage of flamingos stems from another red carotenoid, astaxanthin. The beautiful colors of plants are also responsible for attracting insects and animals for their pollination and seed dispersal [1]. The carotenoid color originates in the structure of multiple conjugated double bonds. This unique structure enables two essential features of carotenoids: the light-harvesting capability and a powerful anti-oxidant effect by the quenching of free radicals, singlet oxygen and reactive oxygen species. In photosynthetic organisms, carotenoids are indispensable for photosynthesis and photo-protection [2]. In non-photosynthetic organisms including animals, the anti-oxidant activity not only protects cells from oxidative damages (e.g., oxidative DNA damage [3]) but can provide additional benefits for humans such as anti-inflammatory and anti-cancer effect [4]. In addition, carotenoids play an important health role as pro-vitamin A compounds. About 30–50 carotenoids are believed to have vitamin A activity including two well-known compounds: β-carotene and α-carotene [2].

Vitamin A includes retinol, retinal and retinoic acid, which are all apocarotenoids. Apocarotenoids are a group of oxidative products of carotenoids. While carotenoids contribute to the visual beauty of flowers and fruits, apocarotenoids are famous for the pleasant aromas and give rise to fragrance and palatable flavors of many flowers and fruits (such as rose, violet, tomato and raspberry) [5–7]. These apocarotenoid aromas, in a similar manner to the colored carotenoids, attract pollinators and promote plant-insect interactions [8]. In addition, some apocarotenoids act as hormones. For example, the plant growth hormone, abscisic acid, has multiple functions in plant development processes including bud dormancy and response to environmental stress and plant pathogens [5]. Strigolactones are another important subclass of apocarotenoids, functioning as shoot-branching inhibitors and promoting the formation of symbiotic association between plants and fungi [9, 10].

Due to the color, aroma, remarkable nutrition and health benefits, carotenoids and apocarotenoids have been widely used in food, feed, nutritional, pharmaceutical and personal care industries. The market demands for carotenoids and apocarotenoids are rising rapidly as increasing clinical research studies report various health and pharmaceutical benefits [11–13]. The global carotenoid market is projected to reach 1.53 billion USD by 2021 [14]. The regular uptake of food with a high content of carotenoids (e.g., β-carotene) or retinoids is vital to alleviate vitamin A deficiency. Vitamin A deficiency can lead to severe aftermath including blindness, decreased immune function and even death [15]. Lutein and zeaxanthin are critical for eye health by preventing age-related macular degeneration [16]. Astaxanthin has even more benefits such as potent anti-oxidant activities, promoting immune response, reducing eye fatigue, enhancing muscle performance and so on [11]. Because of low exceptional fragrance property, α-ionone and β-ionone are widely used in cosmetics such as perfumes [17]. Crocin is another valuable apocarotenoid and is responsible for the red pigmentation of saffron, a high-value spice with retail prices ranging between 2000 and 7000 euros/kg [18].

Despite carotenoids and apocarotenoids being widely distributed in nature, their cellular contents are extremely low. For example, 100 tons of raspberries, or 20 hectares of agricultural area, could only yield 1 g of α-ionone [19]. Similarly, it requires the manual harvest of stigmas from as many as 110,000–170,000 flowers to obtain 1 kg of saffron [20], justifying the high cost of

these molecules. Chemically synthetized carotenoids, despite being less expensive, have been reported to have hazardous effects to human health and are increasingly unpopular with consumers [19]. Currently, microbial-derived commercial carotenoids are those derived from native producer strains which have not been genetically engineered but in some cases have undergone classical mutagenesis followed by selection to screen for improved production characteristics. These include the β-carotene production strains of the microalga *Dunaliella* [21] and the fungus *Blakeslea trispora* [2]. Recent advances in microbial biotechnology have made the microbial production of carotenoids and apocarotenoids potentially more efficient and cost-effective, using metabolic engineering strategies in industrial workhorse strains such as *Escherichia coli* and *Saccharomyces cerevisiae*, for which the fermentation strategies are well established.

To date, 1117 natural carotenoids and apocarotenoids have been reported, which consist of C30, C40, C45 and C50 carotenoids [22]. Among them, C40 carotenoids and their derived apocarotenoids are the most abundant with 1093 different structures. In this chapter, I will cover only a few of the commercially interesting C40 carotenoids and apocarotenoids that will illustrate the challenges and potentials of this biosynthetic alternative supply chain.

2. Biosynthesis of carotenoids and apocarotenoids in nature

To understand how carotenoids and apocarotenoids can be produced in microbes, it is essential to elucidate the biosynthetic enzymes which constitute these metabolic pathways.

Carotenoids are a subclass of terpenoids (or isoprenoids); thus, as other terpenoids, they share the common C5 building blocks, isopentenyl pyrophosphate (IPP) and its isomer dimethylallyl pyrophosphate (DMAPP). In nature, there exist two independent biosynthetic pathways to produce IPP/DMAPP: the mevalonate (MVA) pathway [23] and the 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway, also referred to as the 1-deoxy-D-xylulose 5-phosphate (DXP) or the non-MVA pathway [24].

The MEP pathway starts from the condensation of pyruvate and glyceraldehyde-3-phosphate, which are catalyzed by DXP synthase (*dxs*), to produce DXP, which is subsequently reduced into MEP by DXP reductase (*dxr*). MEP is converted into 4-diphosphocytidyl-2-C-methyl-D-erythritol (CDPME) by CDPME synthase (*ispD*). CDPME is subsequently transformed into 1-hydroxy-2-methyl-2-(E)-butenyl-4-diphosphate (HMBPP) through two intermediates, 4-(cytidine 5′-diphospho)-2-C-methyl-D-erythritol (CDPMEP) and 2-C-methyl-D-erythritol-2,4-cyclodiphosphate (MEC) by CDPME kinase (*ispE*), MEC synthase (*ispF*) and HMBPP synthase (*ispG*), respectively. Finally, HMBPP reductase catalyzes HMBPP into a 5-6:1 ratio of IPP and DMAPP, while IPP isomerase (*idi*) inter-converts IPP and DMAPP to adjust the ratio according to the cellular requirements (**Figure 1**).

In the MVA pathway, two molecules of acetyl-CoA are condensed into one molecule of acetoacetyl-CoA by acetyl-CoA acetyltransferase (*atoB*). Acetoacetyl-CoA is converted into mevalonate via an intermediate (S)-3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) by HMG-CoA synthase and HMG-CoA reductase, respectively. IPP is produced from mevalonate by another three enzymes, mevalonate kinase (*mk*), phosphomevalonate kinase (*pmk*) and

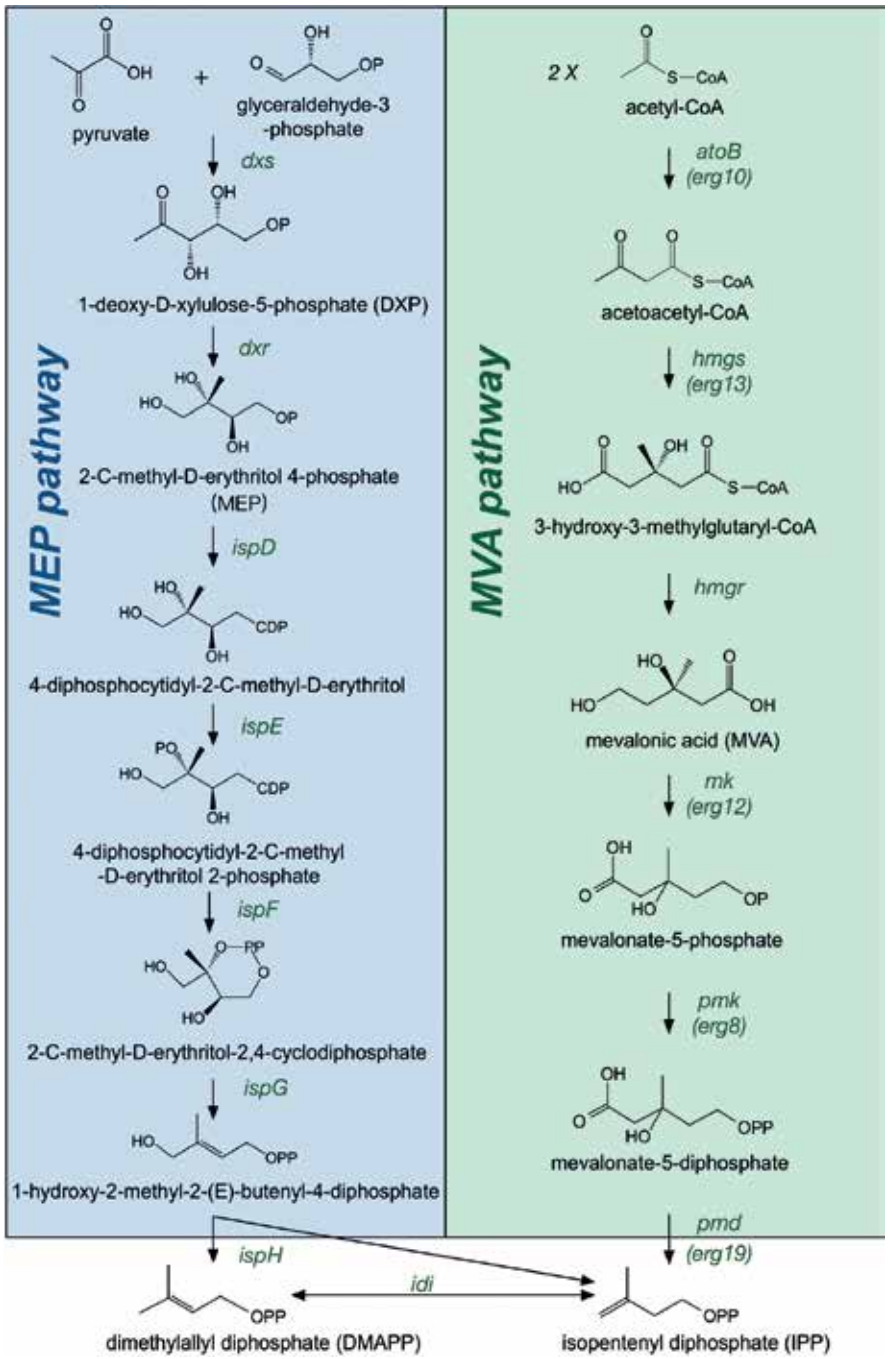

Figure 1. Biosynthetic pathway of terpenoid precursors. Carotenoids are a subclass of terpenoids. In nature, two major biosynthetic pathways of terpenoids exist, one is the 2-C-methyl-D-erythritol 4-phosphate (MEP) pathway, and the other is the mevalonate (MVA) pathway. Abbreviations: *dxs* (DXP synthase), *dxr* (DXP reductase), *ispD* (4-diphosphocytidyl-2-C-methyl-D-erythritol, or CDPME synthase), *ispE* (CDPME kinase), *ispF* (2-C-methyl-D-erythritol-2,4-diphosphate synthase), *ispG* (1-hydroxy-2-methyl-2-(*E*)-butenyl-4-diphosphate synthase), *ispH* (1-hydroxy-2-methyl-2-(E)-butenyl-4-diphosphate reductase), *atoB* (acetoacetyl-CoA thiolase), *hmgs* (hydroxymethylglutaryl-CoA, or HMG-CoA synthase), *hmgr* (HMG-CoA reductase), *mk* (mevalonate kinase), *pmk* (phosphomevalonate kinase), *pmd* (phosphomevalonate decarboxylase), and *idi* (IPP isomerase).

phosphomevalonate decarboxylase (*pmd*). Thus, while the MEP pathway produces mixtures of DMAPP and IPP, the MVA pathway produces only IPP and requires *idi* to generate DMAPP (**Figure 1**).

Most bacteria including cyanobacteria use exclusively the MEP pathway, whereas most eukaryotes and archaea possess only the MVA pathway. Interestingly, plants have both pathways: the MVA pathway located in the plant cytoplasm and the MEP pathway located in plastids. This is consistent with the hypothesis that chloroplasts originate from cyanobacteria endosymbionts [25]. Both pathways have been engineered to produce terpenoids including carotenoids. The MEP pathway has a higher theoretical yield than the MVA pathway [26] due to its adoption of a variety of cofactors (ATP, NADPH, CTP and flavodoxin, etc.) whereas the MVA pathway mainly uses ATP. However, in practice, it is easier to manipulate the MVA pathway and its theoretical yield has been achieved for certain products [27–30]. In contrast, the practical yield of the MEP pathway is often limited by the low activity of ispG and ispH enzymes and their special requirement of iron-sulfur cofactors. To the best of my knowledge, the highest reported yields of terpenoids synthesized by the MEP pathway in literature are less than 20% of its theoretical yield [30].

The two pathway metabolites IPP and DMAPP are condensed to give geranyl diphosphate (GPP, C10) or farnesyl diphosphate (FPP, C15), catalyzed by GPP synthase (*gpps*) or FPP synthase (*fpps*), respectively. Geranylgeranyl diphosphate (GGPP) synthase catalyzes the addition

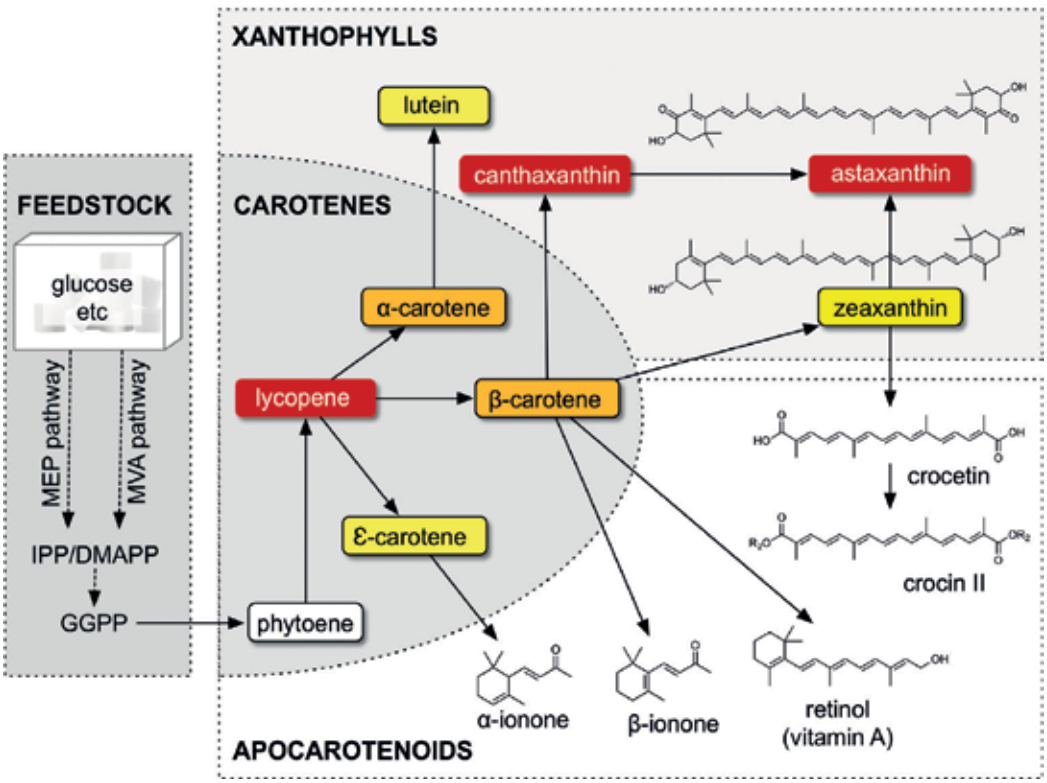

Figure 2. Biochemical pathway of carotenoids and apocarotenoids.

of another IPP into FPP to yield GGPP (C20). Finally, phytoene synthase (*crtB*) catalyzes the first committed step of carotenoid biosynthesis, the formation of one molecule of phytoene (C40) from two molecules of GGPP (**Figure 2**). Phytoene is a colorless acyclic carotene with only three conjugated double bonds. All the C40 carotenoids are derived from phytoene, which accounts for over 90% of total carotenoids to date [22]. Based on molecular structures, carotenoids are classified into two groups: carotenes and xanthophylls. Carotenes are hydrocarbon carotenoids with only carbon and hydrogen atoms (e.g., lycopene and β-carotene), whereas xanthophylls are oxygenated carotenoids by hydroxylation, ketolation and epoxidation (e.g., astaxanthin, lutein, **Figure 2**) [31]. In plants, algae, fungi and bacteria, apocarotenoids are derived from the oxidation of carotenoids or other apocarotenoids with carotenoid cleavage enzymes (such as carotenoid cleavage dioxygenases or CCDs and apocarotenoid cleavage oxygenases or ACOs) [32]. Some apocarotenoid examples are shown in **Figure 2**.

3. Production of carotenoids in engineered microbes

3.1. Biosynthesis of carotenes

As a colorless carotene, phytoene is the common precursor to all the C40 carotenoids (**Figure 2**). It exhibits excellent anti-UV activity [33] and is clinically proved to have activities of skin whitening and wrinkle reduction [34]. Hence, there are increasing cosmetic products developed based on phytoene. Phytoene is an intermediate carotenoid in plants and exists only as a minor product; hence, it is expensive to extract phytoene from plant materials. Consequently, it is promising to engineer microbes to produce higher concentrations of phytoene and more importantly, to produce it at high purity without other carotenoids. By deleting the *crtI* gene, encoding phytoene desaturase (see below), from an engineered lycopene-producing strain of *Escherichia coli* previously developed in our laboratory [19], it was relatively simple to generate strains of producing more than 50 mg/L of high-purity phytoene in simple low-cell density shake flasks [35]. Although this carotene with a high-potential market in cosmetics could be relatively simple to transfer to the industry, this is only just the beginning to attract interest, as witnessed by a French company Deinove (www.deinove.com) [36]. Despite certainly being more efficient than the use of the tomato strain developed to this end, there could still be considerable progress made by optimizing the engineered strains such as that used in our study and coupling this to high cell density fermentation processes to achieve a more cost-effective process.

Lycopene, a red color pigment most commonly associated with tomatoes, belongs to one of the top six commercial carotenoids. It is produced from the dehydrogenation of phytoene catalyzed by different types of phytoene desaturases (*crtI*, *PDS* or *ZDS*, **Figure 2**). Lycopene has been used as animal feed, food coloring and nutritional products. Some clinical studies have suggested that lycopene functions in reducing the risk of prostate cancers [37, 38]. In recent years, multiple research groups reported relatively high concentrations of lycopene produced in *E. coli* and yeasts. In *E. coli*, Kim et al. have used a mixture of carbon sources containing glucose, glycerol and arabinose to produce lycopene at 1.35 g/L [39]. Our laboratory initially optimized the MEP pathway which enabled the *E. coli* strain to produce at 20 mg/g

dry cell weight (DCW) of lycopene [40] and more recently reconstituted the MVA pathway in *E. coli* to produce lycopene in a glucose-defined medium, reaching 1.5 g/L in a simple non-optimized fed-batch process [19]. Xie et al. evolved the bifunctional enzyme crtYB to acquire only the phytoene synthase function. By applying this mutated enzyme and optimizing the copy number of crt genes, the engineered *Saccharomyces cerevisiae* strain produced 1.61 g/L of lycopene [41]. In addition, *Yarrowia lipolytica*, the oleaginous yeast, has also been engineered to produce lycopene but at slightly lower yields [42, 43].

Moving further along the carotenoid biosynthetic pathway, lycopene is usually cyclized into β-carotene or α-carotene by a lycopene cyclase (**Figure 2**). Both α- and β-carotenes are yellow pigments; β-carotene is more commonly marketed, being one of the most important commercial carotenoids. As mentioned earlier, β-carotene is a direct precursor of vitamin A (**Figure 2**). It has been widely used as a colorant, nutritional supplement, animal feed and in pharmaceutical and personal care industries. Chemically synthesized β-carotene is less popular among consumers than that extracted from natural sources or so-called 'bio-based' sources. At the same time, naturally derived β-carotene has gradually taken over the market. Currently, β-carotene is produced mainly in the microalga *Dunaliella* [21] and the fungus *Blakeslea trispora* [2]. As summarized in **Table 1**, many groups have engineered fast-growing microorganisms and achieved high titers of β-carotene. Yang et al. have applied a hybrid MVA pathway in *E. coli* to overproduce β-carotene at 3.2 g/L [44]. Zhao et al. have engineered the central metabolic pathway to increase cofactor supply in an *E. coli* strain, which enabled the strain to produce at 2.1 g/L of β-carotene [45]. *Y. lipolytica* has shown potential as a better host for producing β-carotene; 4 g/L of β-carotene was achieved in an *Y. lipolytica* strain by integrating multiple copies of key enzymes (hmgr in **Figure 1** and the bi-functional enzymes phytoene synthase/lycopene cyclase carRP) [46]. Recently, based on an engineered lipid overproducing strain of *Y. lipolytica*, Larroude et al. have rewired it to produce at 6.5 g/L and 90 mg/g DCW of β-carotene [47]. These results are relatively better than those previously achieved [48–50]. It would not be surprising if some of these examples would lead to the successful commercialization notably of novel β-carotene sources in the near future.

3.2. Biosynthesis of xanthophylls

The modification of carotenes by enzymes such as hydroxylases and ketolases leads to the synthesis of xanthophylls (**Figure 2**). Due to the polarity introduced by oxygen, xanthophylls have different physical properties and physiological activities. For example, unlike carotenes, most xanthophylls do not possess provitamin A activity but do have higher anti-oxidant activities. The reason is that, in addition to the polyene structure, the functional groups of xanthophylls such as keto groups in the β-ionone rings can also quench singlet oxygen resides [31].

Among various xanthophylls, astaxanthin is the most important commercial product. Astaxanthin is a red pigment with numerous health benefits. As a potent anti-oxidant, astaxanthin protects the tissue against UV-light damage [51–53] and exhibits anti-cancer activity [54, 55] and anti-inflammatory properties [56]. In double-blind, randomized controlled trials, astaxanthin lowered oxidative stress in obese subjects and improved cognition and promoted proliferation of nerve stem cells [57]. Astaxanthin also improves integrated immune response [58], reveals anti-aging effects by protecting red blood cells in both aging and young people

No.	Hosts	Carotenes	Titer (mg/L)	Content (mg/g DCW)	Culture conditions	References
1	*Escherichia coli*	Phytoene	50	35	1–2 days, in flasks	[35]
2	*Escherichia coli*	Lycopene	224	34.5	1–2 days, in flasks	[48]
3	*Escherichia coli*	Lycopene	/	20	1–2 days, in flasks	[40]
4	*Escherichia coli*	Lycopene	1500	35	2 days, in bioreactors	[19]
5	*Escherichia coli*	Lycopene	1350	32	2 days, in bioreactors	[39]
6	*Saccharomyces cerevisiae*	Lycopene	1610	24.4	5–6 days, in bioreactors	[41]
7	*Saccharomyces cerevisiae*	Lycopene	1650	54.6	5–6 days, in bioreactors	[49]
8	*Yarrowia lipolytica*	Lycopene	/	16	7–8 days, in flasks	[42]
9	*Yarrowia lipolytica*	Lycopene	213	21.1	10 days, in bioreactors	[43]
10	*Blakeslea trispora*	β-Carotene	5600	/	7 days, in bioreactors	[50]
11	*Escherichia coli*	β-Carotene	2100	60	3–4 days, in bioreactors	[45]
12	*Escherichia coli*	β-Carotene	3200	/	2–3 days, in bioreactors	[44]
13	*Yarrowia lipolytica*	β-Carotene	6500	90	5–6 days, in bioreactors	[47]
14	*Yarrowia lipolytica*	β-Carotene	4000	50	10–11 days, in bioreactors	[46]

Table 1. Microbial production of carotenes in literature.

[59, 60] and relieves eye fatigue especially beneficial for persons spending too much time on the computer and smartphones [61]. In addition, astaxanthin supplement can prevent atherosclerotic cardiovascular disease [62, 63] and diabetes [64, 65]. More importantly, besides all these benefits, astaxanthin is clinically proven to be safe for human and animals. Therefore, astaxanthin has been widely used in fish feeding, food, nutritional, medicinal and cosmetic industries. The current global annual market of astaxanthin is around 250 tons worth $447 million [66], and it is growing rapidly. Synthetic astaxanthin, like β-carotene, is less popular with consumers and yields a mixture of three isomers, RR, RS and SS, at the ratio of 1:2:1 and appears to be less available for assimilation than the natural forms. Astaxanthin produced by the microalga *Haematococcus pluvialis* has a higher cost and lower purity than synthetic astaxanthin so additional work is required before good natural astaxanthin can be marketed effectively. Furthermore, astaxanthin in microalgae, shrimp and fish exists as an ester form rather than in the free form, which limits its nutraceutical applications.

Due to the wide application of astaxanthin, many researchers have been working hard to engineer microbes to produce high titer and yield of astaxanthin. It is not trivial to optimize the biotransformation of β-carotene to astaxanthin as the biosynthetic pathway is rather complex with many intermediates and a complex network of enzymatic reactions [67]. By screening different β-carotene hydroxylases and ketolases, there has been success to improve astaxanthin production from sub-milligram to milligram per gram DCW [67, 68]. Further optimization of the metabolic pathway leading to astaxanthin synthesis has led to improved yields which are now promising for commercialization. For example, Zhou et al. developed a *S. cerevisiae* strain that overproduced astaxanthin at 47.2 mg/L and 8.1 mg/g DCW, where they used a direct evolution approach to generate a triple mutant of beta-carotene ketolase with higher activity [69]. Lin et al. integrated a multicopy of the key biosynthetic genes of astaxanthin (*hpchyb* and

No.	Hosts	Carotenoids	Titer (mg/L)	Content (mg/g DCW)	Culture conditions	References
1	*Saccharomyces cerevisiae*	Astaxanthin	/	4.7	3–4 days in flasks	[72]
2	*Saccharomyces cerevisiae*	Astaxanthin	/	0.029	5 days in flasks	[73]
3	*Escherichia coli*	Astaxanthin	/	2.64	2 days in flasks	[74]
4	*Escherichia coli*	Astaxanthin	/	0.31	2 days in flasks	[68]
5	*Escherichia coli*	Astaxanthin	2.1	1.41	2 days in flasks	[75]
7	*Escherichia coli*	Astaxanthin	2.9	1.99	2 days in flasks	[67]
8	*Corynebacterium glutamicum*	Astaxanthin	/	1.6	2 days in flasks	[76]
9	*Xanthophyllomyces dendrorhous, previously as Phaffia rhodozyma*	Astaxanthin	1.6	0.29	3 days in flasks	[77]
10	*Xanthophyllomyces dendrorhous*	Astaxanthin	/	9.0	8 days in flasks	[78]
11	*Xanthophyllomyces dendrorhous*	Astaxanthin	561	5.0	4–5 days in bioreactors	[79]
12	*Saccharomyces cerevisiae*	Astaxanthin	47.2	8.1	3–4 days in flasks	[69]
13	*Kluyveromyces marxianus*	Astaxanthin	/	9.90	3 days in bioreactors	[70]
14	*Yarrowia lipolytica*	Astaxanthin	54.6	3.5	3–4 days in plates	[66]
15	*Escherichia coli*	Astaxanthin	320	15.0	2 days in bioreactors	[71]
16	*Xanthophyllomyces dendrorhous*	Zeaxanthin	10.8	0.5	7.5 days in flasks	[80]
17	*Escherichia coli*	Zeaxanthin	/	11.9	2 days in bioreactors	[81]
18	*Escherichia coli*	Zeaxanthin	722	23.2	2.5 days in bioreactors	[82]

Table 2. Microbial production of astaxanthin and zeaxanthin in literature.

bkt) into the *Yarrowia lipolytica*. As a result, they were able to achieve about 9.97 mg/g DCW of astaxanthin [70]. By developing and using an efficient multidimensional heuristic process and colorimetric medium screening approach, our laboratory has achieved one of the best results of astaxanthin using *E. coli*, 320 mg/L and 15 mg/g DCW [71]. As summarized in **Table 2**, the engineered *S. cerevisiae* [69], *Y. lipolytica* [66], *Kluyveromyces marxianus* [70] and *E. coli* [71] have produced promisingly high titers and yields of astaxanthin. The recently achieved titers and yields [66, 70, 71] are from 10-fold to 100-fold higher than those previously reported in *S. cerevisiae* [72, 73], *E. coli* [74, 75], *Corynebacterium glutamicum* [76] and *Xanthophyllomyces dendrorhous*, previously as *Phaffia rhodozyma* [77–79].

Zeaxanthin is another important xanthophyll with high commercial values. Lutein, an isomer of zeaxanthin, is typically found in plants (such as corn), whereas zeaxanthin is present in cyanobacteria and some non-photosynthetic bacteria [2]. Although both lutein and zeaxanthin are used as colorants and potentially in pharmaceutical and nutraceutical industries, the demand for alternative sources of zeaxanthin is more urgent than lutein. Till now, *X. dendrorhous* has been engineered to produce 10 mg/L of zeaxanthin [80]. The first attempt to produce zeaxanthin in *E. coli* achieved 11.9 mg/ g DCW in bioreactors [81]. A few years later, the same group applied a dynamic control system to *E. coli* in which 720 mg/L of zeaxanthin was produced [82] (**Table 2**).

4. Production of apocarotenoids in engineered microbes

As shown in **Figure 2**, carotenoids can be further converted into apocarotenoids by CCDs or other oxygenases. Here, three apocarotenoids of high commercial values are highlighted here. Retinol, or vitamin A, is one of the most important apocarotenoids to humans. Retinol exhibits an essential function in vision, bone development and also promotes skin health as an anti-oxidant [83]. The other two are aromatic molecules, α-ionone, which naturally exists in raspberry, and β-ionone, which is found in many flowers, for example, rose, osmanthus and violet [84]. The chemical synthesis of these three molecules is not very difficult and contributes significantly to the current market share. However, consumers prefer natural derivatives and are willing to pay higher prices for natural ingredients [19]. As mentioned in the introduction, the extremely low concentrations in natural plant materials make their extraction an extremely expensive process. Consequently, the fermentation of engineered microbes is a promising alternative route.

4.1. Biosynthesis of retinol or vitamin A

As an important nutritional compound and an active cosmetic ingredient, retinol market size is estimated at 1.6 billion dollars [85]. Jang et al. pioneered retinol production in metabolically engineered *E. coli* [85]. Unlike carotenoids that are stored intracellularly in the lipid structures of microbes, apocarotenoids are smaller and thus can pass the cell membrane into the culture media. Consequently, a two-phase culture system was applied to capture extracellular retinol and improve its production by minimizing its degradation [85]. The same group later identified a gene (*ybbo*) that has retinal reductase activity that converts retinal into retinol.

Consequently, overexpression of the YBBO enzyme improved the final yield (76 mg/L) and purity (88%) of retinol in the final products [86]. Based on our lycopene chassis strain, we developed a 'plug-n-play' system that could easily adapt our *E. coli* strain into different apocarotenoids, such as α-, β-ionones and retinol [19] with promising results obtained (**Figure 3**).

4.2. Biosynthesis of α- and β-ionone

Both α-ionone and β-ionone have exceptional aroma activities as their odor threshold is at the sub-ppb range [7, 87]. Hence, they have been widely used as fragrance molecules in cosmetics and perfumes. As consumers prefer natural ingredients, the market demand for natural ionone is increasing dramatically. In addition, there is a chiral center for α-ionone. Natural α-ionone from plants (such as raspberry) is (R)-(+)-(E)-alpha-ionone. In contrast, synthetic α-ionone has two isomers (R and S). The R-enantiomer has a unique and strong floral flavor and aroma, described as a violet-like, fruit-like or raspberry-like flavor, while the S-enantiomer is woody or β-ionone like. Lashbrooke et al. did a proof-of-principle production of α-ionone at about 300 ng/L [88]. By coupling the modular metabolic engineering approach and enzyme engineering methods (N-terminal truncation and protein fusion), we developed an *E. coli* strain to produce 'natural identical' α-ionone at almost 500 mg/L, about 1400 times higher than that previously reported [19] (**Table 3**). Similarly, Phytowelt (www.phytowelt.com), a German company, has also developed an *E. coli*-based process to produce α-ionone, demonstrating that it has attracted more commercial interest.

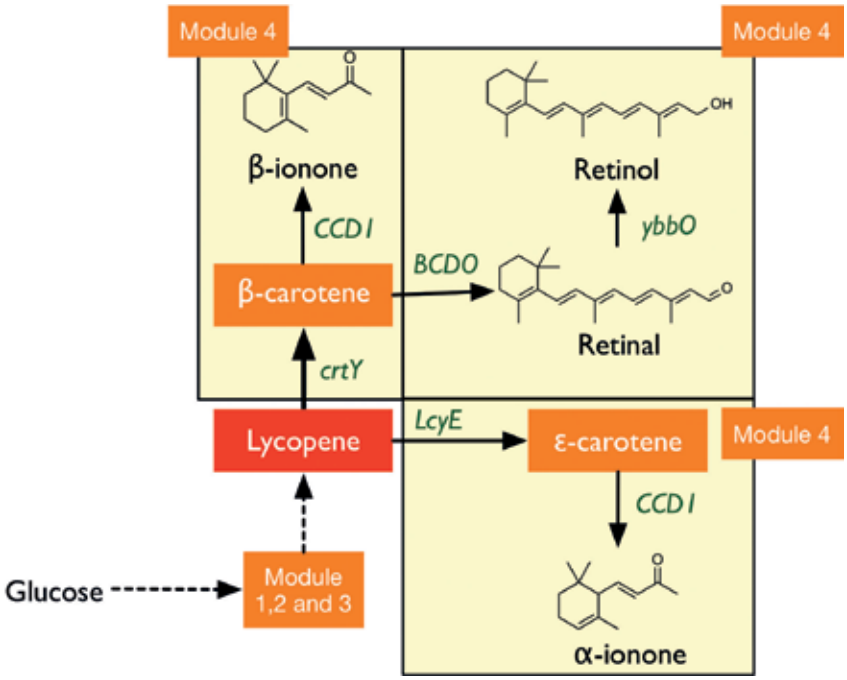

Figure 3. A 'plug-n-play' platform for biosynthesis of apocarotenoids. Adapted from author' s paper [19]. crtY—lycopene beta-cyclase; CCD1—carotenoid cleavage dioxygenase; BCDO (or blh)—β-carotene dioxygenase; ybbO—NADP+-dependent aldehyde reductase.

No.	Hosts	Apocarotenoids	Titer (mg/L)	Specific titer (mg/g DCW)	Culture conditions	References
1	*Escherichia coli*	Retinol	54	6.3	2–3 days in flasks	[85]
2	*Escherichia coli*	Retinol	76	9.8	2–3 days in flasks	[86]
3	*Escherichia coli*	Retinol	28	10.0	2 days in flasks	[19]
4	*Saccharomyces cerevisiae*	β-Ionone	0.22	/	2–3 days in flasks	[87]
5	*Saccharomyces cerevisiae*	β-Ionone	6	1.0	2–3 days in bioreactors	[90]
6	*Escherichia coli*	β-Ionone	500	16.0	2 days in bioreactors	[19]
7	*Escherichia coli*	α-Ionone	340 ng/L	/	2 days in flasks	[88]
8	*Escherichia coli*	α-Ionone	480	7.0	2 days in bioreactors	[19]

Table 3. Microbial production of retinol, α- and β-ionones in literature.

Although several groups have attempted to produce β-ionone using yeast or *E. coli*, their yields are relatively low. Simkin et al. firstly engineered *E. coli* cells to synthesize β-ionone but with only detectable trace amounts being reported [89]. Beekwilder et al. engineered *Saccharomyces cerevisiae* for the production of β-ionone; however, the titer achieved was only 0.22 mg/L [87]. López et al. inserted extra copies of geranylgeranyl diphosphate synthase gene and CCD1 gene from the plant *Petunia hybrid*, which enabled their *S. cerevisiae* strain to produce about 6 mg/L of β-ionone when grown in a bioreactor [90]. To date, the best-reported β-ionone strain was from our laboratory, where the engineered *E. coli* strain produced 500 mg/L of β-ionone [19] (**Table 3**).

5. Challenges and potential for the commercialization of microbial production of carotenoids and apocarotenoids

In general, the chief challenge for commercializing microbial production of chemicals is relatively high cost. The cost depends mainly on titer, rate (or productivity) and yield (or 'TRY') [91]. Hence, researchers are inventing and exploring different approaches to engineer microbes to obtain TRY figures of merit. Until then, it would not be cost effective or competitive to other sources (such as chemical synthesis). The good news is that carotenoids and apocarotenoids are high-value specialty chemicals; thus, their requirements for commercialization are less stringent as compared to fuels and commodity chemicals. For example, the current processes of β-carotene production in microalga *Dunaliella* [21] and the fungus *Blakeslea trispora* [2] are already profitable. Many recent cases of microbial production of carotenoids have reached

TRY figures [46, 47, 71] higher than existing processes. It is not surprising that some of them will be translated into more cost-effective industrial processes. More importantly, scientists and engineers are working together to continue improving microbial strains and fermentation processes. Breakthrough by innovation and collective knowledge will markedly reduce product cost and make it more competitive. In addition, the recent trend of consumers' preference to 'natural' or 'bio-based' ingredients will make microbial-derived carotenoids and apocarotenoids more appealing.

6. Conclusion

Amid diverse natural products, carotenoids and apocarotenoids are particularly interesting. This is not only due to their bright color and pleasant fragrances but also their light-harvesting capability, the electron/energy transferring ability, the potent anti-oxidant properties, the hormone function, vitamin A activity and numerous other health benefits to both human and other life forms on the Earth. Increasingly, clinical studies have supported the concept that the regular uptake of carotenoids can prevent many serious diseases. The list of benefits and applications keeps growing and with the market for commercial exploitation it can be confidently expected to increase. In light of this and the extremely low levels found in plant materials, it is urgent to find solutions enabling these valuable molecules to be supplied in a sustainable and cost-effective manner. In the past decade, the metabolic engineering of microorganisms has progressed remarkably for the production of carotenoids and apocarotenoids. Some of these processes are being commercialized already but the scope to further extend this family of molecules is high, adding an increasingly solicited pipeline of natural products to compete with chemical synthesis.

Acknowledgements

This work was supported by the research funding of Biotransformation Innovation Platform (BioTrans), Agency for Science, Technology and Research (A*STAR), Singapore. The author appreciates Dr. Nic Lindley, Ms. Sudha Devi Manbahal Shukal and Ms. Chin Chin Lim in BioTrans for invaluable advice.

Author details

Congqiang Zhang

Address all correspondence to: zcqsimon@outlook.com

Biotransformation Innovation Platform (BioTrans), Agency for Science, Technology and Research (A*STAR), Singapore

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Chapter 6

Carotenoids in Raw Plant Materials

Lucia Maria Jaeger de Carvalho,
Gisela Maria Dellamora Ortiz,
José Luiz Viana de Carvalho and
Alcides Ricardo Gomes de Oliveira

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.78677

Abstract

Carotenoids are rich sources of pro-vitamin A. These compounds are usually obtained from pumpkins (*Cucurbita maxima, C. pepo, and C. moschata*), as well as orange and yellow sweet potatoes. Carotenoids are C40 tetraterpenoids, which stand out for their antioxidant activity. Among them are carotenes (very apolar carbon and hydrogen molecules, like lycopene, β-carotene, α-carotene) and oxygenated derivatives and xanthophylls composed of oxygenated functions (less apolar molecules such as lutein, zeaxanthin, cryptoxanthin). β-Carotene is the most commonly found carotenoid, accounting for 25–30% of the total carotenoid content of plants. It is also the most active carotenoid, with the highest bioconversibility in the human body. β-Carotene is a suppressor of tumorigenesis in the skin, lung, liver, and colon, promoting the cessation of the cell multiplication cycle. Thermal processing can affect the sensory characteristics and the antioxidant compounds, altering the antioxidant potential of foods. Time, temperature, and style of cooking are determinant conditions for the increase or decrease of total antioxidant activity. The biological activity of carotenoids depends on their bioaccessibility and solubilization in the gastrointestinal tract. The purpose of this chapter is to offer information about some raw plant materials containing carotenoids.

Keywords: raw plant materials, yellow bitter and sweet cassava, sweet yellow and orange potato, pumpkin, carotenoids

1. Introduction

Some carotenoids are rich sources of pro-vitamin A and carrots (*Daucus carota* L.) were the first raw plant material source of carotenoids, which were isolated in 1831 [1], while the first separation and purification procedures were performed by Tswett [2]. Pumpkins

(*Cucurbita maxima, C. pepo,* and *C. moschata*), as well as orange and yellow sweet potatoes, are usually good sources of carotenoids including α and β-carotene. On the other hand, the yellow sweet and bitter cassava (*Manihot esculenta*) roots were studied by Oliveira et al. [3], which found lower contents of β-carotene. To improve these contents, many efforts have been made through the biofortification.

Carotenoids are chemically defined as C40 tetraterpenoids (naturally occurring hydrocarbons and their derivatives), obtained by the union of eight isoprenoid (C5) units of five carbon atoms [4].

The purpose of this chapter is to offer some information about some raw plant materials containing high and lower carotenoid contents.

2. Raw plant materials

2.1. Carotenoids

Due to their intense and striking colors, carotenoids have always been a subject of interest of scientists. The first report of isolation of these substances is from 1831, from the carrot (*Daucus carota* L.), which also gave rise to the name of the class, derived from the English "carrot" [1]. The first separation and purification processes of carotenoids are attributed to the Russian botanist Tswett [2], who invented the liquid chromatography for the separation of carotenoids from colored leaves [5]. Tswett also gave rise to the concept of a family composed of many compounds, carotenoids, among them carotenes (composed only of very apolar carbon and hydrogen molecules, like lycopene, β-carotene, α-carotene) and oxygenated derivatives and xanthophylls composed of oxygenated functions, such as ketones, ethers, hydroxides, epoxides, methoxides, or carboxylic acids (less apolar molecules such as lutein, zeaxanthin, cryptoxanthin).

Carotenoids have aliphatic or acyclic structures (open chain) and alicyclic or cyclic structures (closed chain). Cyclic or alicyclic carotenoids can be monocyclic (when there is a ring) or bicyclic (when there is more than one ring) [5]. β-Carotene is the most commonly found of these compounds, accounting for 25–30% of the total carotenoid content of plants [6] or even more in some of them.

Due to their double-bonded conjugate system, carotenoids exhibit ultraviolet and visible spectral absorption characteristics, and most have maximum absorption at three wavelengths, resulting in a spectrum consisting of three peaks. The greater the number of conjugated double bonds in the carotenoid, the greater the spectrum wavelength [7].

According to Krinsky et al. [8], at least seven conjugated double bonds are necessary for a carotenoid to have color, as in the case of ζ-carotene, which gives a yellow color to passion fruit. As the conjugate system is extended, the color also intensifies. Therefore, lycopene with 11 conjugated double bonds gives rise to the red color of tomatoes (*Solanum lycopersicum*). Cyclization places the double bonds within the rings, outside the plane of those of the polyene

chain, decreasing their coloration. Thus, γ-carotene, with a double conjugated ring-located bond, is reddish orange, while β-carotene with two of these ring bonds is orange (carrot), although both have conjugated double bonds as does lycopene.

The detection of carotenoids, after separation by liquid chromatography methods, occurs in a characteristic absorption zone between 400 nm and 500 nm wavelength; the detection in cis- or *Z*- isomers usually occurs between 330 and 340 nm. The intensity of absorption is affected by the solvent or the composition of the mobile phase used in the analysis [7].

Carotenoids consist of a wide range of substances, with great structural diversity and varied functions, of which more than 600 have already been identified and had their chemical structures elucidated. They are probably the most occurring pigments in nature, and the many different colors we see are the result of the presence and combination of these different compounds [8].

The official nomenclature of carotenoids was established in 1974 by the International Union of Pure and Applied Chemistry (IUPAC) and the International Union of Biochemistry [9].

They stand out commercially in the production of rations for breeding sites (fish, crustaceans, and poultry) and are used as food dyes and in aromas. In addition to the food industry, carotenoids play an important role in the pharmaceutical industry due to their nutritional and functional properties, as precursors of vitamin A, antioxidant activity, among others [10].

They can be divided into two groups: carotenes and xanthophylls. Carotenes are pure hydrocarbons, which have an orange to red coloration. This group includes β-carotene, α-carotene, ζ-carotene, δ-carotene, and lycopene. β-Carotene is the most commonly found of carotenes, accounting for 25–30% of the total carotenoid content of plants and even more in some of them [6].

It is also the most active carotenoid, with the highest bioconversibility in the human body, covering 15–30% of all serum carotenoids. β-Carotene is described as a suppressor of tumorigenesis in the skin, lung, liver, and colon, promoting the cessation of the cycle of cell multiplication. It also shows a suppression activity superior to that promoted by α-carotene [11]. Lycopene does not have pro-vitamin A activity but is considered as the carotenoid with the highest singlet oxygen sequestration capacity, possibly due to the presence of two unconjugated double bonds, which make it more reactive [12, 13].

Thermal processing can lead to important changes in the sensory characteristics and the content of antioxidant compounds, altering the antioxidant potential of foods. Conditions such as time, temperature, and style of cooking are determinants for the increase or decrease of the total antioxidant activity [14].

The biological activity of carotenoids depends on their bioaccessibility and solubilization in the gastrointestinal tract. Due to their lipophilic nature, these compounds do not disperse well in the aqueous medium of the gastrointestinal tract. Therefore, it is important to analyze how food matrix and processing affect their bioaccessibility. Rodriguez-Roque et al. [15] formulated beverages with mixtures of fruit juices and water, milk, and soy applying three treatments: high intensity pulse electric fields, high pressure processing, and thermal treatment, to

evaluate bioaccessibility of carotenoids and on lipophilic antioxidant activity. Bioaccessibility of carotenoids was reduced after all treatments, except for *cis*-violaxanthin and neoxanthin, which increased 79% in beverages treated with high intensity pulse electric fields and high pressure processing. The thermal treatment presented worst decreasement of the bioaccessibility in 63%. High-intensity pulse electric fields and high pressure processing can be considered as promising technologies to obtain nutritive and functional beverages.

2.2. Orange flesh sweet potato (*Ipomoea batatas*)

Sweet potato (*Ipomoea batatas* (L.) Lam.) is a typical vegetable of tropical and subtropical countries (Mozambique), being one of the most consumed in Brazil, mainly by low-income populations. It occupies the sixth position among the most cultivated vegetables in the country, being an excellent source of energy and protein for the families of small farmers in the Northeast and South regions. It presents no difficulties in its cultivation, besides being very resistant to the dry climate, having a great capacity of adaptation, and is one of the largest energy producers per unit of area and time. Another advantage is that it requires low financial investments, with high returns having great importance in animal feed, industrial production of flour and starch and can be cooked in different styles in boiling water [16, 17].

It is a herbaceous crop with extensive growth in tropical and subtropical regions of the world, being important in many developing countries. Archeological, linguistic, and historical evidences establish that sweet potatoes originated in the region of Central and South America. The ability of this crop to adapt to a wide variety of climatic conditions allowed for its development in tropical and temperate regions of Africa, Asia, and the Americas. Compared to other crops, sweet potatoes are able to grow at an accelerated rate under various environmental conditions and are highly adaptable under marginal growing conditions. It has a short production cycle, high nutritional value, and sensory versatility in terms of color, flavor, and texture [18].

In Brazil, sweet potato presents low average productivity due to the occurrence of pests and diseases, inadequate production technology, and the absence of selected cultivars. However, improved productivity can be easily achieved through the use of seedlings from disease-free matrices obtained from tissue culture laboratories [17].

Its forms of consumption are the same as those of other sweet potato cultivars, as well as having similar production techniques. Its planting can be carried out at any time of the year, provided that the minimum local temperature in the period is equal to or above 15°C [16].

In the form of flour, *Beauregard* sweet potato is a possible total or partial substitute of wheat flour in recipes, allowing its introduction in school meals and in basic baskets [16]. Its high content of β-carotene, on average 115 μg.g^{-1} of root, gives intense orange coloration to its pulp [16]. Carvalho et al. [19] found 111.44 μg g^{-1} of total carotenoids, 104.17 μg g^{-1} of β-carotene, 13-*cis* isomer 3.38 μg g^{-1} and 9-*cis* isomer 1.49, respectively, in the raw roots in the same variety, somewhat lower than was found before.

Many authors evaluated the β-carotene content in orange sweet potato cultivars and found variable contents: Hangenimana et al.—79.84 μg g^{-1} [20], Takahata et al.—187.00 μg g-^{1} [21], Lako et al.—150.0 μg g^{-1} [22], Kidmose et al.—108.0 μg g^{-1} [23], Teow et al.—226.00 μg g^{-1} [24], Wu et al.—84.00 μg g^{-1} [25], and Failla et al.—281.00 μg g-^{1} [26].

The average found for the centesimal composition in orange flesh sweet potato was moisture—83.91 g 100 g^{-1}, ash—0.52 g 100 g^{-1}; protein—0.69 g 100 g^{-1}; lipids—0.10 g 100 g^{-1}; carbohydrates—13.42 g 100 g^{-1}, respectively, with a caloric value of 57.34 kcal 100 g^{-1} [19].

The orange sweet potato pulps have the potential to be used as food-based supplements to reduce vitamin A deficiency since β-carotene is one of the carotenoids with pro-vitamin A activity for human diet, exerting important functions in human physiology, acting as antioxidants, as protective pigments of the human retina, and as precursors of retinoids that influence gene expression [27].

Orange-fleshed sweet potato (OFSP) is a carotenoid-rich vegetable. Thermal treatment to process sweet potatoes can decrease the contents of these compounds in foods, reducing their bioactive properties. Raman spectroscopy can be employed as a fast tool in food analysis, especially to detect low concentrations of carotenoids and to monitor their degradation profile along time. Sebben et al. [28] evaluated two drying methods, hot air and microwave in a rotating drum, coupled to quantitative Raman spectroscopy. A 50% decrease in the carotenoid contents were found for both types of drying methods. The results were reproducible. The best linear correlations were $R^2 = 0.90$ for hot air and 0.88 for microwave dried samples, respectively.

Vitamin A deficiency (VAD) is a public health problem in some regions of Brazil. Enhancement of the use of orange-fleshed sweet potatoes as a pro-vitamin A source can be a strategy for prevention of this deficiency. Berni et al. [29] compared the pro-vitamin A contents, vitamin A equivalencies and β-carotene (βC) bioaccessibilities of two varieties (*Beauregard* and Amelia) of home-cooked orange sweet potatoes and two commercial ones. Pro-vitamin A carotenoid content in home cooked *Beauregard* variety was higher than in Amelia variety and in commercial products for babies. All-*trans*-βC was the most abundant carotenoid in all samples (raw, cooked, and commercial) as expected. Boiling and frying decreased total β-carotene. According to them, a portion of 100 g fresh weight of *Beauregard* contained over 100% of the estimated average requirement for children and women and up to 92% estimated average requirement for lactating women. The efficiency of micellarization of all-*trans*-βC after the in vitro digestion was relatively low (4–8%) and significantly less than for *cis*-isomers, the amounts of *trans*-βC captured into micelles from boiled *Beauregard* and fried Amelia varieties were higher than in micelles obtained from the digestion of commercial ones. Bioaccessibility of pro-vitamin A carotenoids in the micelle fraction of digested OFSP was confirmed in assays of Caco-2 human intestinal cells. They suggested that agricultural development of these two varieties: Amelia and *Beauregard* (biofortified), riches in *trans*-βC, and the improvement of home cooking styles can be strategies to increase the consumption of this food to reduce VAD in Brazil.

Islam [27] analyzed total carotenoids and *trans* and cis-β-carotene in different varieties of raw and cooked orange-fleshed sweet potato (OFSP) aiming to reduce VAD using plant-based foods. Intravarietal difference in carotenoids as well as in *trans* and *cis*-β-carotenes were found both in raw and boiled potatoes. Carotenoid content was higher in raw potatoes compared to boiled samples from the same variety, as expected by solids dissolution. Amongst the varieties, Kamalasundari (BARI SP-2) contained the highest amount of carotenoids both in raw and boiled samples. The β-carotene was significantly higher in Kamalsundari and BARI SP-5

varieties, while *trans*-β-carotene was found the major carotenoid in all of the raw potatoes. Boiling resulted in an increase in *cis*-β-carotene and a decrease in the *trans*-isomer contents. The use of Kamalsundari and BARI SP-5 orange-fleshed sweet potatoes was proposed as potential food-based supplements to reduce vitamin A deficiency.

Sweet potatoes have been the aim of research over the years due to their functional and nutritional properties. Carbohydrates, proteins, lipids, carotenoids, anthocyanins, phenolic acid conjugates, and minerals constitute versatile nutrients in different parts (tubers, leaves, stems, and stalks) of sweet potato. The unique composition of sweet potato provides various beneficial effects such as antioxidant, hepatoprotection, anti-inflammatory, anticancer, antidiabetic, antimicrobial, anti-obesity, and antiaging activities. Factors which affect the nutritional composition and bio-function of sweet potatoes include the varieties, parts of the plants, extraction time and solvents, post-harvest storage and processing. Differences between the *in vitro* and *in vivo* assays employed for bio-function evaluation also lead to variations in results from different studies, which makes direct comparisons inadequate or difficult. Leaves, stems, and stalks from sweet potatoes are still commercially underutilized. Results from several studies point out that sweet potato can be developed as a sustainable crop for different nutritionally enhanced and value-added food products aiming at the promotion of human health [30].

Sweet potato (*Ipomoea batatas* (L.) Lam) is one of the most popular and ancient roots in Brazil, which is consumed cooked, baked or as sweets, cooked in boiling water. Cooking can result in physicochemical transformations which modify its nutritional properties. Vizzotto et al. [31] evaluated physicochemical characteristics, bioactive compounds, and the antioxidant activity of 12 genotypes of raw and roasted sweet potato, with different pulp colors: cream pulp, orange pulp, and purple pulp. Total soluble solids, acidity, sugars, carotenoids, anthocyanins, phenolic compounds contents, and antioxidant activity show a wide variation of these parameters for both forms of preparation. Antioxidant activity varied considerably amongst the genotypes, from 210.29 to 7870.57 μg trolox equivalent.g^{-1} for pulps *in natura* and from 773.26 to 17,306.22 μg trolox equivalent g^{-1} for baked pulps. Contents of soluble solids, acidity, sugars, and bioactive compounds as well as total antioxidant activity were higher while the levels of carotenoids were lower in baked sweet potatoes than *in natura*. Genotype and color of sweet potatoes influenced their chemical composition. Cultivars Amelia and *Beauregard* stood out with respect to the amount of soluble solids and carotenoids, respectively. Selections of purple have to be recommended as sources of anthocyanins. Thermal processing influenced the concentration of antioxidant compounds and affected some of the physicochemical characteristics.

Yellow sweet potato is mostly produced in small scale by farmers. It is a source of energy and carotenoids in the human diet; however, it is a highly perishable crop. To increase its industrial use, yellow sweet potato flour was produced for use in bakery products. Nogueira et al. [32] evaluated the technological quality and the carotenoid content in sweet breads produced by replacing wheat flour with 0, 3, 6, and 9% yellow sweet potato flour. The increase in yellow sweet potato flour concentrations in bread resulted in a decrease of specific volume and firmness and an increase in water activity, moisture, orange coloring, and carotenoids. Storage led to the most significant changes after the 5th day, with a reduction in intensity of

the orange color. The β-carotene content varied from 0.16 to 0.47 μg g^{-1} in breads with yellow sweet potato flour. The results suggest that the use of yellow sweet potato in breads can be beneficial for consumers' health and for the agricultural business as well.

2.3. Pumpkins (*Cucurbita moschata*)

A great number of pumpkin varieties, each of which contains a different amount of carotenoids, are cultivated worldwide [33]. In several Brazilian regions, *C. moschata* cultivars are known to contain a particularly high amount of α- and β-carotene. The β-carotene has 100% pro-vitamin A activity, and α-carotene has 53% pro-vitamin A activity [34–37].

Carotenoids have antioxidant activity, but few are converted in retinol (an active form of vitamin A) by the human body. Among more than 600 carotenoids which have pro-vitamin A activity, the most known are α- and β-carotene, and these are susceptible to degradation (isomerization and oxidation during the cooking process).

There are various studies about carotenoids from pumpkins, mainly β-carotene, and the large diversity of landraces and cultivars, among them the *Cucurbita maxima, Cucurbita moschata,* and *Cucurbita pepo.* The differences in carotenoids and β-carotene contents in these three species are important in order to choose the best one for cultivation and biofortification [19]. For instance, studies carried out by Carvalho et al. [19] revealed a large range of contents of carotenoids among samples of the same species of *Cucurbita moschata*. The total carotenoids content varied from 124.87 to 557.20 μg g^{-1} and others were above 60 μg.g^{-1}.

Priori et al. [38] evaluated the genetic variability for the synthesis of bioactive compounds, total phenolic compounds, carotenoids, antioxidant activity, and minerals of 10 accesses of pumpkin (*C. moschata*) landraces. They found a high genetic variability for the synthesis of phenolic compounds, carotenoids, antioxidant activity, and minerals, with the most promising C52 and C389 accessions, due to their high levels of total carotenoids.

2.4. Yellow sweet cassava (*Manihot esculenta*)

The manioc plant (*Manihot esculenta*, Crantz) belongs to the Euphorbiaceae family being native to South America, cultivated by the Indians responsible for its dissemination. The Portuguese spread it to other continents, especially Africa and Asia The plant is a bush of bulky roots, leaves petiolated and yellowish chalice flowers, arranged in panicles. Its tubercle is also known as cassava, aipim, castelinha, macacheira, cassava, sweet cassava, and cassava according to the regions where it is cultivated [13].

In Brazil, there are about 1200 varieties, classified as bitter or sweet according to its hydrocyanic acid content. Originated from South America, manioc (*Manihot esculenta*, Crantz), present in the indigenous culture and other ancient populations, has its historical importance because it has been the main energetic source for several generations of these peoples. It is still one of the main energy foods in the African, Latin American, and Asian continents, to about 500 million people, especially in developing countries [39].

Cassava is easily adapted to different types of soil and climate, usually grown on a small scale with little or no technology adoption, basically using family workers. In the case of Brazil, it is widespread throughout the region bounded by the geographic tropics. The world production has increased over the last decades, due to factors such as genetic improvement, use of technology in planting, and expansion of cultivated areas. However, the expansion of the areas remains centralized in the countries that have a tradition in the planting of this culture [3, 40].

The economies of African countries, the largest producer continent, and Latin America are based on the exploitation of the primary sector. Therefore, countries seek to increase production of crops that are strategic for maintaining the economy and serving the domestic market, raising production more than in other continents (Africa) or maintaining production (Latin America) in the last decades. On the other hand, Thailand, located on the second largest cassava (Asian) producer continent, is the largest cassava root exporting country.

The African continent, the world's largest producer of cassava roots, does not have countries that excel in the export trade of the product, prevailing the service in the domestic market, indicating that the crop is produced mainly by small producers, in precarious production systems, with little or no application of modern management and fertilization technology. Brazilian production stood out among the 80 cassava producing countries, reaching around 13% of world production [41], demonstrating that there is still room for growth of Brazilian production if modernization of plantation using technology and improvement is implemented. Ten states produced about 80% of the Brazilian production, with Pará and Bahia accounting for 36% of this total and Paraná, Rio Grande do Sul and Maranhão, 26%. The other five: Amazonas, Minas Gerais, Ceará, São Paulo and Mato Grosso do Sul contributed 18% of this production [41]. Overall, 62% of the national production comes from the North and Northeast regions. Brazil has encouraged the production and commercialization of agricultural products inside the country, through government financing programs such as the National Family Agriculture Program (PRONAF). This program is based on support for rural development, on the strengthening of family agriculture and its organizations, for example, cooperatives, as a segment that generates jobs, incomes, and increases the nutritional quality of these populations [42].

In the last 10 years, efforts were made to identify new varieties of able yellow cassava to improve the nutritional quality for the populations with malnutrition problems, situated in the tropics, particularly in the Brazilian northeast, where the cassava constitutes one of the main cultivations and almost the only source of nutrients. The cassava culture of yellow coloration can be an excellent source of carotenoids, precursors of vitamin A such as α and β-carotene.

The total and α and β-carotene in raw varieties of bitter and sweet yellow cassava as well as in cooked ones were evaluated by Oliveira et al. [40]. A total of 28 varieties were analyzed: 12 in bitter, 11 in sweet yellow cassava, and 5 other varieties of bitter yellow cassava. The variability among the varieties of bitter yellow cassava revealed higher total carotenoids content compared to sweet yellow ones. However, the proportion of β-carotene in relation to the total carotenoids content was larger in the varieties of sweet yellow cassava. The bitter yellow cassava roots presented a variation in the total carotenoid content from 1.97 to 16.33 μg g^{-1} and β-carotene from 1.37 to 7.66 μg g^{-1}, respectively. The isomers 13, 9-Z and all-*trans*-β-carotene were found in all varieties, being all-E-β-carotene the most abundant one [43–45].

3. Conclusions

The evaluation of β and α-carotene and total carotenoids content in cultivars, accesses, biofortified, and landraces of sweet potatoes, pumpkins, and yellow sweet and bitter cassava is very important to obtain plant raw materials with high contents of carotenoids that can be used for cultivation and minimize hunger in the low-income populations of all ages around the world [46, 47].

Acknowledgements

The authors wish to thank the Carlos Chagas Filho, Embrapa – Food Technology, BioFORT Network and Harvest*Plus* for the financial support.

Conflict of interest

There is no interest conflicts.

Author details

Lucia Maria Jaeger de Carvalho[1]*, Gisela Maria Dellamora Ortiz[1],
José Luiz Viana de Carvalho[2] and Alcides Ricardo Gomes de Oliveira[1]

*Address all correspondence to: luciajaeger@gmail.com

1 Faculty of Pharmacy, Federal University of Rio de Janeiro, Rio de Janeiro, Brazil

2 Embrapa, Food Technology, Rio de Janeiro, Brazil

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Chapter 7

Astaxanthin as a Modifier of Genome Instability after γ-Radiation

Denys Kurinnyi, Stanislav Rushkovsky,
Olena Demchenko and Mariya Pilinska

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.79341

Abstract

The chapter is devoted to study the effects of astaxanthin on the frequency of chromosomal aberrations and the level of DNA damages in human peripheral blood lymphocytes under ionizing radiation exposure in vitro. To achieve the purpose of the research, a combination of classical cytogenetic methods (G_0- and G_2-radiation sensitivity assays) and method of single-cell electrophoresis (comet assay) was used. The specificity of the modifying effect of astaxanthin on radiation-induced genomic injuries depending on the stage of the cell cycle had been determined. Significant weakening of the negative effect of ionizing radiation on the G_0 stage and the absence of a radioprotective effect on the S and G_2 stages of the cell cycle may be associated with activation by astaxanthin of apoptosis in irradiated cells with a critically high level of the genome damages. The research results not only testify about strong radioprotective effect of astaxanthin but also demonstrate the feasibility of the parallel use of cytogenetic and molecular genetic methods to assess the impact as mutagens as well as factors that modify the effect of mutagens on genome stability.

Keywords: astaxanthin, lymphocytes, γ-radiation, DNA breaks, chromosomal aberrations

1. Introduction

The ecological situation that arose from nuclear accidents in Chornobyl and Fukushima, constant expansion of usage of the ionizing radiation in industry and medicine, and the threats of nuclear terrorism especially aggravated in the last decade are risk factors for the growth of radiation burden on human populations. The abovementioned conditions require the search

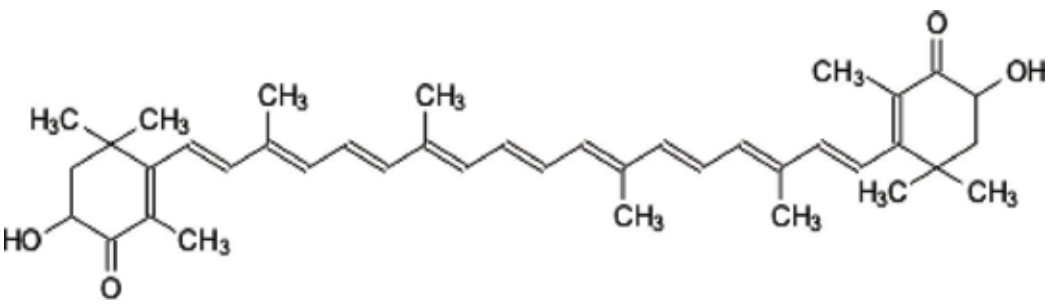
for new safe and effective radioprotectors, preferably of natural origin, for prevention and treatment of radiation-induced damages in humans, especially which cause genome alterations and cancer. For that purpose, carotenoids, due to its chemical and biological properties, are the most promising substances [1].

Astaxanthin is a carotenoid of xanthophyll group, and it is one of the most common red pigments of algae, yeasts, krill, shrimps, crayfish, trout, and salmon [2]. It is known that astaxanthin is the most powerful antioxidant, which has the ability to scavenge free radicals in tens of times higher than α-tocopherol or β-carotene [3], and has anti-inflammatory [2], immunomodulating [4], and anticarcinogenic [5–7] effects.

Since 2015, we have started the investigation of the radioprotective effects of astaxanthin studying parameters of genome damages in human somatic cells. In this chapter, we have concentrated on physicochemical properties of astaxanthin and its biological effects with the main focus on the data from our investigations concerning the impact of astaxanthin on radiation-induced genome damages in human somatic cells and have discussed eventual mechanisms of its action.

2. Physicochemical properties and peculiarities of biological action of astaxanthin

Astaxanthin is a secondary carotenoid, which belongs to the group of xanthophylls and has two additional oxygen atoms on each benzene ring in comparison with β-carotene. This gives astaxanthin a rich red color and greatly increases its antioxidant properties. Unlike β-carotene, astaxanthin is not a vitamin A precursor [8].



Empirical formula: $C_{40}H_{52}O_4$

Molar weight: 596.84 g/M

In contrast to primary carotenoids, which are associated with the structural and functional components of the photosynthetic apparatus, secondary carotenoids, which include astaxanthin, are in the cell in oil droplets, and their main function is to form a protective layer to prevent the damages, which are provoked by stress conditions [9, 10].

Because the astaxanthin molecule contains conjugated double bonds, hydroxyl and keto groups, it has both lipophilic and hydrophilic properties [11]. Astaxanthin has two chiral centers and can exist in three different stereoisomers—3S, 3′S; 3R, 3′S; and 3R, 3′R. The probability of obtaining these isomers of astaxanthin in the process of chemical synthesis is 1:2:1 [12, 13].

Nowadays natural astaxanthin mainly derived from microalgae (hyperproducer *Haematococcus pluvialis*), yeast (*Phaffia rhodozyma*) and animal-consumers included a number of small marine crustaceans (Euphausiacea) and the salmon family (Salmonidae*)* [2]. Microalgae *Haematococcus pluvialis* produces astaxanthin mainly 3S, 3′S stereoisomeric form; precisely, such molecular structure is considered the most valuable [14].

As shown in experiments *in vitro*, astaxanthin effectively protects cells from nonspecific oxidation by quenching singlet oxygen, effectively inhibits lipid peroxidation in biological samples, and owing to the capture of free radical prevents or stops the chain reaction of oxidation [2, 15, 16]. In addition to direct protective effect, astaxanthin inhibits the activation of the H_2O_2-mediated transcription of the factor NF-kB (the nuclear factor "kappa-b"—a universal transcription factor) that controls the expression of heme oxygenase 1 (HMOX1), one of the markers of oxidative stress, and nitric oxide synthase (iNOS) [17, 18]. Astaxanthin blocks the cytokine production declined by modulating the expression of protein tyrosine phosphatase 1 [18].

Experiments on the determination of astaxanthin toxicity showed a high level of safety—LD_{50} was not established after single administration of substance to rats. The studies confirmed the absence of histopathological changes and the dose-effect dependence upon oral administration of astaxanthin in doses ranging from 4.161–17.076 to 465.0–557.0 mg/kg per day [19].

The accumulated published data have shown the multifaceted positive effect of astaxanthin in mammals by reducing the manifestations of oxidative stress, including during inflammation processes; it can prevent the development of atherosclerotic cardiovascular diseases and participate in the regulation of lipid and glucose metabolism [19–23].

These properties of astaxanthin primarily attributed to its ability to exhibit activity both at the level of the cell membrane and in the area of the cytoplasm, thus affecting the flow of intracellular processes [2]. Due to these unique properties, astaxanthin exhibits significantly higher biological activity in comparison with other antioxidants [24].

Thus, the above data indicate that astaxanthin complies with all the requirements that apply to radioprotectors (low toxicity, high antiradical and antioxidant activity, the ability to act both at the membrane level and in the intracellular space). These properties of astaxanthin suggest that it may have antimutagenic activity and, as consequence, radioprotective effect on the human genome.

3. Investigation of radioprotective properties of astaxanthin

Since 2015, we examined the possibility of modification by astaxanthin and the negative effects of ionizing radiation on the human blood lymphocyte genome in vitro. The decrease in the intensity of radiation-induced genome damages on the chromosomal and molecular

levels was selected as an indicator of radioprotective effect of astaxanthin. The studies were conducted using a combination of the methods of classical cytogenetic analysis (G_0-radiation sensitivity assay and G_2-radiation sensitivity assay) and the method of single-cell electrophoresis (comet assay) [25–29].

The parallel application of two methodological approaches for such a study greatly expanded the experimental possibilities. Thus, due to cytogenetic methods, the state of the chromosomal apparatus of the cell (frequency of different types of chromosome aberrations) is clearly visualized starting from the 48 h of cultivation. The comet electrophoresis is highly sensitive and provides the ability to determine the relative levels of single- and double-strand DNA breaks in individual cell. When conducting cell electrophoresis, the DNA migrates into the agarose gel, forming a structure that resembles a comet (**Figure 1**), and the use of the comet assay can simultaneously estimate the effect of both mutagenic and antimutagenic factors on the stability of the human somatic cell genome, starting from 0 h of cultivation [30, 31]. In addition, the use of single-cell electrophoresis makes it possible to determine the effectiveness of the reparation systems and to assess the correctness of the operation of control mechanisms at checkpoints between all stages of the cell cycle (G_1–S, S–G_2, G_2–M). Moreover, an important feature of the comet assay is the identification of cells in which the apoptosis program has begun or has already been implemented [32–34].

In cells with a lack or a low level of damages, the "tail" is formed also by the release of DNA loops into the gel. Because in the cell during realization of the apoptotic process genomic fragmentation of the high level occurs, a massive yield of DNA fragments into agarose gel is observed (**Figure 1**), and "comets" have the typical elongated "tail" part.

To quantify the migration of DNA into the agarose gel, two indices are used: the percentage of DNA in the "tails" and tail moment (TM). TM simultaneously which takes into account both the

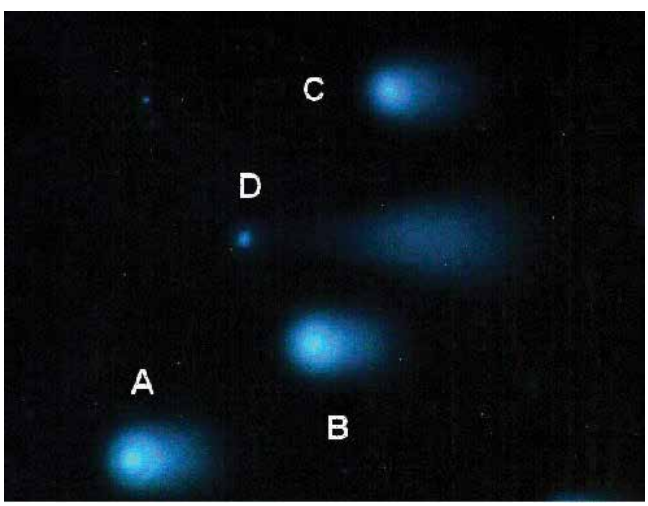


Figure 1. Examples of "comets" obtained in the experiment: (A, B, C) The "comets" arisen from cells with a low level of DNA breaks and (D) "atypical comet" (apoptotic cell) [28].

amount of DNA and the length of the "tail" (TM = "tail" length multiplied by the percentage of DNA in the "tail") is more informative and calculated automatically during the computer analysis.

3.1. The impact of astaxanthin on the level of radiation-induced chromosomal aberrations in human lymphocytes

To evaluate the possible mutagenic activity of astaxanthin, it was tested at concentrations of 2.0, 10.0, 20.0, and 40.0 μg/ml in the culture of human peripheral blood lymphocytes. In the cytogenetic assay, it was found that the frequencies of aberrant cells and the levels of chromosomal aberrations under the astaxanthin exposure in vitro in all tested concentrations did not differ from the corresponding background cytogenetic parameters ($p > 0.05$) [25].

To determine the optimal working concentration of astaxanthin for further research of its radiomodifying capacity, a pilot study of its impact on the culture of human peripheral blood lymphocytes is exposed in vitro to gamma quanta in a dose of 1.0 Gy on G_0 phase of the first mitotic cycle (**Figure 2**).

It is established that astaxanthin in all tested concentrations significantly ($p < 0.01$) reduced the frequencies of radiation-induced chromosome aberrations, but the effectiveness of its modifying action depended on its concentration in the irradiated culture.

The maximum radioprotective effect of astaxanthin (the most effective drop in the frequency of cytogenetic markers of radiation exposure) was observed after administration of astaxanthin before irradiation of cultures at concentrations of 20.0 and 40.0 μg/ml (7.69 ± 1.74 and 7.72 ± 1.80 per 100 cells, respectively). These concentrations did not affect the mitotic activity of the lymphocyte culture, had no mutagenic effect on non-irradiated cells, and effectively (to ~ 70%) reduced the level of aberrant metaphases and the frequency of cytogenetic markers of radiation exposure. So long as significant difference between the values that characterize carotenoid activity in these concentrations ($p > 0.05$) was not observed, for the further studies of the radiomodifying capacity of astaxanthin, the concentrations of 20.0 μg/ml were chosen.

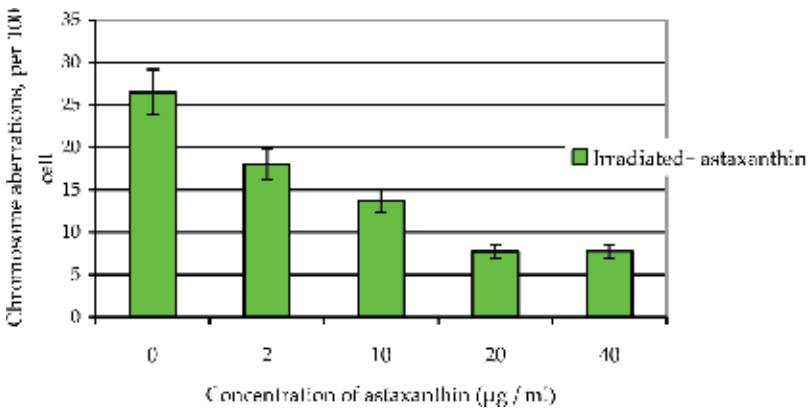


Figure 2. Selection of the optimal concentration of astaxanthin to study its modifying effect on the γ-irradiated culture of human blood lymphocytes.

To analyze the possible dependence of radioprotective properties of astaxanthin from the stage of the mitotic cycle on which the cells were exposed to ionizing radiation, lymphocyte cultures were irradiated at 0, 40, and 46 h, corresponding to G_0, S, and G_2 stages of the first cell cycle. Astaxanthin was added to cultures of lymphocytes at least an hour before irradiation. The obtained data are presented in the **Table 1**.

After irradiation of lymphocyte culture in a dose of 1.0 Gy on the G_0 stage of the cell cycle, the effect of astaxanthin resulted in a significant reduction of the radiation-induced cytogenetic effect, namely, a decrease of almost in 3.5 times both the mean frequency of the aberrant metaphases and the level of chromosome aberrations—up to 7.82 ± 0.72% and 8.48 ± 0.75 per 100 cells, respectively—and exclusively due to aberrations of chromosome type (**Table 1**). The antimutagenic activity of astaxanthin was characterized by significant ($p < 0.001$) decrease in the frequency of classical unstable cytogenetic markers of radiation exposure—dysenteric and ring chromosomes (up to 2.37 ± 0.41 and 0.43 ± 0.18 per 100 metaphases, respectively), as well as the total level of simple acentrics—free double fragments, and acentric rings (up to 4.74 ± 0.62 per 100 metaphases) (**Table 1**, **Figure 3**).

In contrast to the modifying activity shown by astaxanthin in lymphocyte cultures irradiated on the G_0 phase of the cell cycle, the addition of carotenoid on the G_2 phase did not change as the total average frequency of radiation-induced chromosomal damages (72.35 ± 1.17 and 71.54 ± 1.34 per 100 metaphases, respectively, $p > 0.05$) as the spectrum of chromosome aberrations (**Figure 4**). Among chromosomal damages, dominated aberrations of chromatid type represented by single fragments and chromatid exchanges with the total average frequency 58.42 ± 1.47 per 100 metaphases did not differ from such (58.32 ± 1.34 per 100 metaphases) in exposed cultures without adding astaxanthin. Aberrations of the chromosome type were mainly represented by free double fragments; the average group frequencies of it did not differ between themselves (13.12 ± 1.00 and 14.03 ± 0.91 per 100 metaphases, respectively).

In much the same way, astaxanthin did not exhibit modifying effect on radiation-induced cytogenetic effects in lymphocyte cultures irradiated on the S stage of the cell cycle. The total mean group frequencies of radiation-induced chromosomal damages were 19.57 ± 1.11 and 18.46 ± 1.15 per 100 metaphases in exposed cultures without and with the previous addition of astaxanthin, respectively. Among the chromosomal damages, simple aberrations prevailed (single and double fragments) in both variants of the experiment (**Table 1**, **Figure 4**).

Thus, due to the use of cytogenetic methods, the following important aspects of the astaxanthin modifying action were established:

1. The effectiveness of astaxanthin has a dependence on the stage of the cell cycle on which lymphocytes were irradiated.
2. The radioprotective effect of astaxanthin is realized in cells exposed only on G_0 stage of the mitotic cycle which manifests in lowering the frequency of chromosome-type aberrations for the induction of which a large number of double-stranded DNA breaks as the error of repairing of such damages are needed, which permit to suggest the impact of carotenoid on cells with the high level of genomic instability.

	Frequency of the aberrant Metaphases	Chromosome aberrations (per 100 cell)	Frequency of chromosome aberrations								
			Chromatid type			Chromosome type					
			Single fragments	Chromatid exchanges	Total	Double fragments	Dysenteric	Centric rings	Abnormal monocentric	Acentric rings	Total
Unirradiated culture	2.52 ± 0.34	2.57 ± 0.35	1.60 ± 0.28	0.00	1.60 ± 0.28	0.96 ± 0.21	0.00	0.00	0.01 ± 0.01	0.00	0.97 ± 0.22
G_0 (1.0 Gy)	22.93 ± 1.19	24.55 ± 1.22	1.54 ± 0.35	0.00	1.54 ± 0.35	6.47 ± 0.70	12.80 ± 0.95	2.76 ± 0.47	0.49 ± 0.20	0.49 ± 0.20	23.02 ± 1.20
G_0 (1.0 Gy + A)	7.82 ± 0.72	8.48 ± 0.75	0.72 ± 0.23	0.00	0.72 ± 0.23	4.67 ± 0.57	2.37 ± 0.41	0.43 ± 0.18	0.22 ± 0.13	0.07 ± 0.07	7.76 ± 0.71
S (1.0 Gy)	18.30 ± 0.97	19.57 ± 1.11	7.94 ± 0.67	2.27 ± 0.33	10.21 ± 0.37	9.36 ± 0.67	0.00	0.00	0.00	0.00	9.36 ± 0.67
S (1.0 Gy + A)	16.92 ± 1.12	18.46 ± 1.15	6.15 ± 0.67	2.30 ± 0.33	8.45 ± 0.67	10.01 ± 1.0	0.00	0.00	0.00	0.00	10.01 ± 1.0
G_2 (1.0 Gy)	47.06 ± 1.31	72.35 ± 1.17	56.04 ± 1.30	2.28 ± 0.39	58.32 ± 1.29	13.76 ± 0.90	0.27 ± 0.51	0.00	0.00	0.00	14.03 ± 0.91
G_2 (1.0 Gy + A)	46.72 ± 1.48	71.54 ± 1.34	56.47 ± 1.47	1.95 ± 0.41	58.42 ± 1.47	12.94 ± 1.0	0.18 ± 0.41	0.00	0.00	0.00	13.12 ± 1.0

Table 1. Comparison of the mean group values of cytogenetic parameters in irradiated in vitro in dose 1.0 Gy human lymphocyte cultures on G_0, S, and G_2 stages of the cell cycle and under the joint action of γ-radiation and astaxanthin in concentration 20.0 μg/ml.

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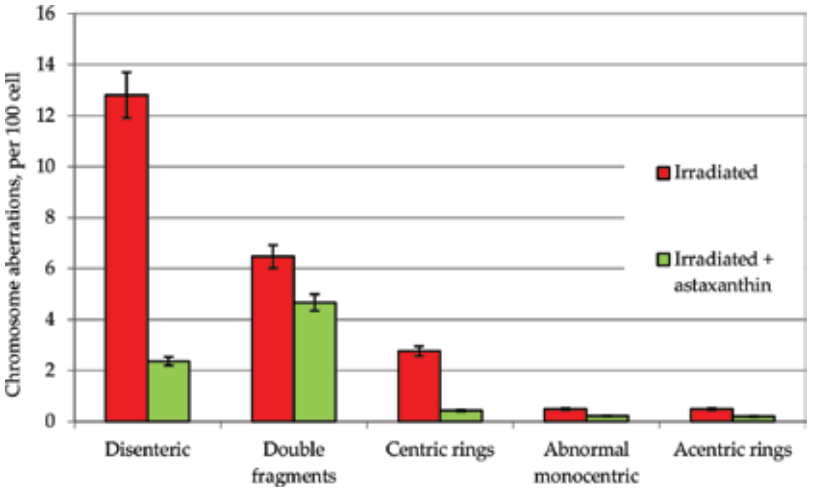

Figure 3. Change in the frequency and spectrum of chromosome-type aberrations under joint action of astaxanthin in the concentration of 20.0 μg/ml and γ-radiation in a dose of 1.0 Gy on the G_0 stage of the cell cycle.

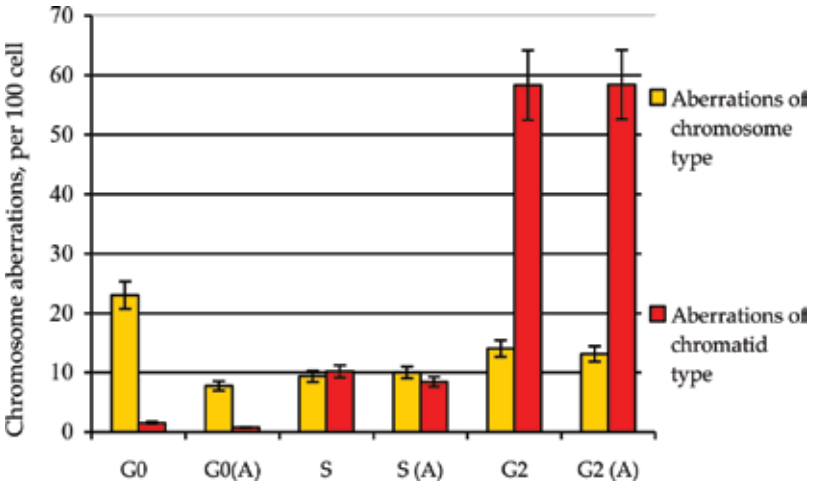

Figure 4. Frequencies and spectra of radiation-induced chromosome aberrations under γ-radiation exposure in dose of 1.0 Gy in vitro and astaxanthin in concentration of 20.0 μg/ml on different stages of the cell cycle. G_0, S, and G_2 (cell cultures irradiated without astaxanthin) and G_0 (A), S(A), and G_2(A) (cell cultures irradiated with supplemented 20.0 μg/ml astaxanthin).

3.2. The impact of astaxanthin on the level of DNA damages in human lymphocytes

For evaluation of the relative level of DNA damages (single- and double-strand DNA breaks), the method of single-cell gel electrophoresis (comet assay) in neutral condition was used. As a parameter of DNA breakage, the TM computed as the %DNA in the comet tail multiplied by the tail length was chosen. For comet assay, we used peripheral blood lymphocytes without

culturing (0 h) and from 48 human-PBL cultures. Some cultures were exposed to γ-ray (emitter IBL-237C, dose rate 2.34 Gy/min) in dose 1.0 Gy at 0, 40, and 46 h of cultivation. Non-irradiated cultures were used as experimental control. Those times were chosen by the reason that lymphocytes, which we can see after 48 h of cultivation on their metaphase stage, are at G_0 (0 h), S (40 h), and G_2 (46 h) phases of the cell cycle. Astaxanthin in the final concentration 20.0 μg/ml, which was defined during our cytogenetic study, was added to the cultures of lymphocytes before irradiation.

Similarly to our cytogenetic data, no significant changes in DNA breakage were detected in non-irradiated samples supplemented with astaxanthin compared with untreated lymphocytes both after 0 and 48 h of cultivation (**Table 2**). This confirms our suggestion that astaxanthin in chosen concentration has no mutagenic activity.

As can be seen from the **Table 2** and **Figure 6**, after γ-irradiation of lymphocytes in dose 1.0 Gy at G_0 phase of the cell cycle, significant increasing in TM was detected (from 2.80 ± 0.54 to 6.55 ± 1.82, $p < 0.05$ and from 4.07 ± 0.60 to 12.86 ± 0.74, $p < 0.05$, after 0 and 48 h of cultivation, respectively).

The effect of astaxanthin on irradiated cells manifested in significant ($p < 0.001$) decrease in the average level of DNA damages in lymphocytes from cultures irradiated at G_0 nearly to the value of non-irradiated control both after 0 and 48 h of cultivation (TM = 3.74 ± 0.82 and 5.27 ± 1.77, respectively) (**Figure 5**).

As expected, significant increase in the level of DNA breaks was detected in lymphocytes after γ-irradiation at 40 h of cultivation (**Table 3**). The mean value of TM was equal to 7.45 ± 0.36 in irradiated and 4.07 ± 0.60 in lymphocytes from intact cultures ($p < 0.01$). Astaxanthin in concentration 20 μg/ml significantly ($p < 0.01$) decreased the DNA damages in lymphocytes from cultures irradiated at 0 and 40 h of incubation nearly to the level of non-irradiated control (TM = 5.27 ± 1.77 and 4.79 ± 0.23, respectively). The treatment of cells with astaxanthin resulted in statistical significant decrease of radiation-induced DNA damages (TM = 3.21 ± 0.48, $p < 0.05$ compared with irradiated samples) likewise after irradiation of lymphocytes at G_0 phase of the cell cycle.

Similar results were obtained after treatment at 46 h of incubation (**Table 4**). Irradiation of lymphocyte cultures at G_2 phase of the cell cycle led to a large amount of DNA breaks and, as outcome, to material increase in TM value (12.06 ± 1.88, $p < 0.001$). The effect of astaxanthin

Treatment	0 h	48 h
	Tail moment (X ± Se)	Tail moment (X ± Se)
Control	2.80 ± 0.54	4.07 ± 0.60
Supplementation with astaxanthin	3.55 ± 1.37	5.93 ± 0.93
Irradiation	6.55 ± 1.82	12.86 ± 0.74

Table 2. The impact of γ-irradiation at G_0 phase and astaxanthin supplementation on DNA damages in human lymphocytes after 0 and 48 h of cultivation.

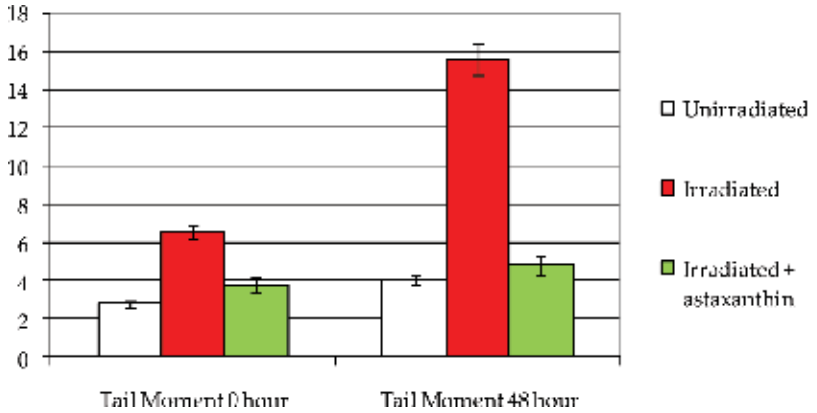

Figure 5. The relative levels of DNA damages irradiated at G_0-phase human lymphocytes non-supplemented or supplemented with astaxanthin cultures.

Treatment	Tail moment	
	X	±Se
Control	4.07	0.60
Control + astaxanthin	5.93	0.93
Irradiation at S phase	7.45	0.36
Irradiation at S phase + astaxanthin	4.79	0.23

Notes: X, mean value; Se, standard error.

Table 3. The tail moment values in human blood lymphocytes after γ-radiation exposure and under combined action of radiation and astaxanthin at S phase of the cell cycle.

Treatment	Tail moment	
	X	±Se
Control	4.07	0.60
Control + astaxanthin	5.93	0.93
Irradiation at G_2 phase	12.06	1.88
Irradiation at G_2 phase + astaxanthin	8.96	2.39

Notes: X, mean value; Se, standard error.

Table 4. The tail moment values in human blood lymphocytes after γ-radiation exposure and under combined action of radiation and astaxanthin at G_2 phase of the cell cycle.

supplementation was somewhat not only weaker than in previous experiments but also significant compared to cultures irradiated on G_2 stage (TM = 8.96 ± 2.39 and 12.06 ± 1.88, respectively, $p < 0.05$).

Our data suggested that astaxanthin decreased the rate of radiation-induced DNA breaks in human lymphocytes regardless of the phase of the cell cycle when the irradiation was performed. However, this conclusion is not consistent with our cytogenetic results: it was observed that astaxanthin is able to decrease frequency of radiation-induced chromosome aberration only if cells were irradiated at G_0 phase of the cell cycle.

For more detailed analysis, we have studied the frequency distribution of individual cells depending on their levels of DNA damages. According to TM, the sampling of "comets" from control variants was divided into ten groups of 10% each. The established values of deciles (TMs were 0.81, 1.28, 1.81, 2.69, 3.80, 5.07, 6.48, 10.19, 15.98) were chosen as boundary indices to form ten groups of cells from irradiated cultures treated or not by astaxanthin and to estimate percentage of "comets" that have TM within the appropriate range. If the value of TM was equal to the boundary index, then "comet" was referred to the next group. The results are shown on **Figure 6**.

When lymphocytes were irradiated at the G_0 phase of the cell cycle, after 48 h of incubation, the increase in the average TM level was caused exclusively by growth of the frequency of the "comets" from the tenth group (TM > 15.98) (**Figure 6A**), which indicates accumulation of cells with a large number of DNA damages with time. Irradiation at the 40th hours of cultivation (**Figure 6B**) resulted in increased levels of the "comets" that belonged to groups 8 and 9 (TM from 6.48 to 15.98). After radiation exposure at 46 h of incubation (**Figure 6C**), the increment of last three groups of "comets" (TM > 6.48) was observed.

It is noteworthy that γ-radiation exposure at 40 and 46 h of incubation did not cause decrease in the frequency of group 1, which includes the "comets" with the smallest DNA release into the "comet" tail (TM from 0 to 0.81). Probably, this situation reflects not so much on the existence in lymphocyte cultures of the populations of radiation-resistant and/or fully recovered cells, as the presence of heavily damaged cells in which the checkpoint has acted on the S phase of the cell cycle, because if the cells are in this phase, then significant decrease of DNA exit under the neutral conditions of electrophoresis is observed [35, 36]. This opinion is confirmed by the lack of increase in frequency of "comets" from the tenth group after radiation exposure at 40 h of cultivation: most of blast-transformed lymphocytes must be on S phase, and cells with the very high level of DNA damages cannot pass S/G_2 checkpoint, and, as a result, they are delayed on this phase.

The supplementation with astaxanthin resulted in significant reduction in the levels of "comets" that belonged only to the ten groups after irradiation at 0 (from 25.07 ± 2.25 to 8.96 ± 1.74%, $p < 0.001$) and at 46 h of cultivation (from 22.38 ± 1.77 to 10.45 ± 1.18%, $p < 0.001$) and groups 9 and 10 after radiation exposure at 40 h of incubation (from 16.56 ± 1.72 to 6.69 ± 1.06, $p < 0.001$ and from 8.60 ± 1.30 to 3.25 ± 0.75%, $p < 0.01$, respectively).

It is known that astaxanthin reveals apoptotic activity in experiments with different cultures of cancer cells [6, 33]. In our studies, the decrease in the frequency of highly damaged cells as a result of astaxanthin treatment may also be caused by activation of apoptotic processes.

The comet assay allowed not only estimating the relative level of DNA damages but also determining the intensity of apoptotic processes [37, 38]. For this purpose simultaneously

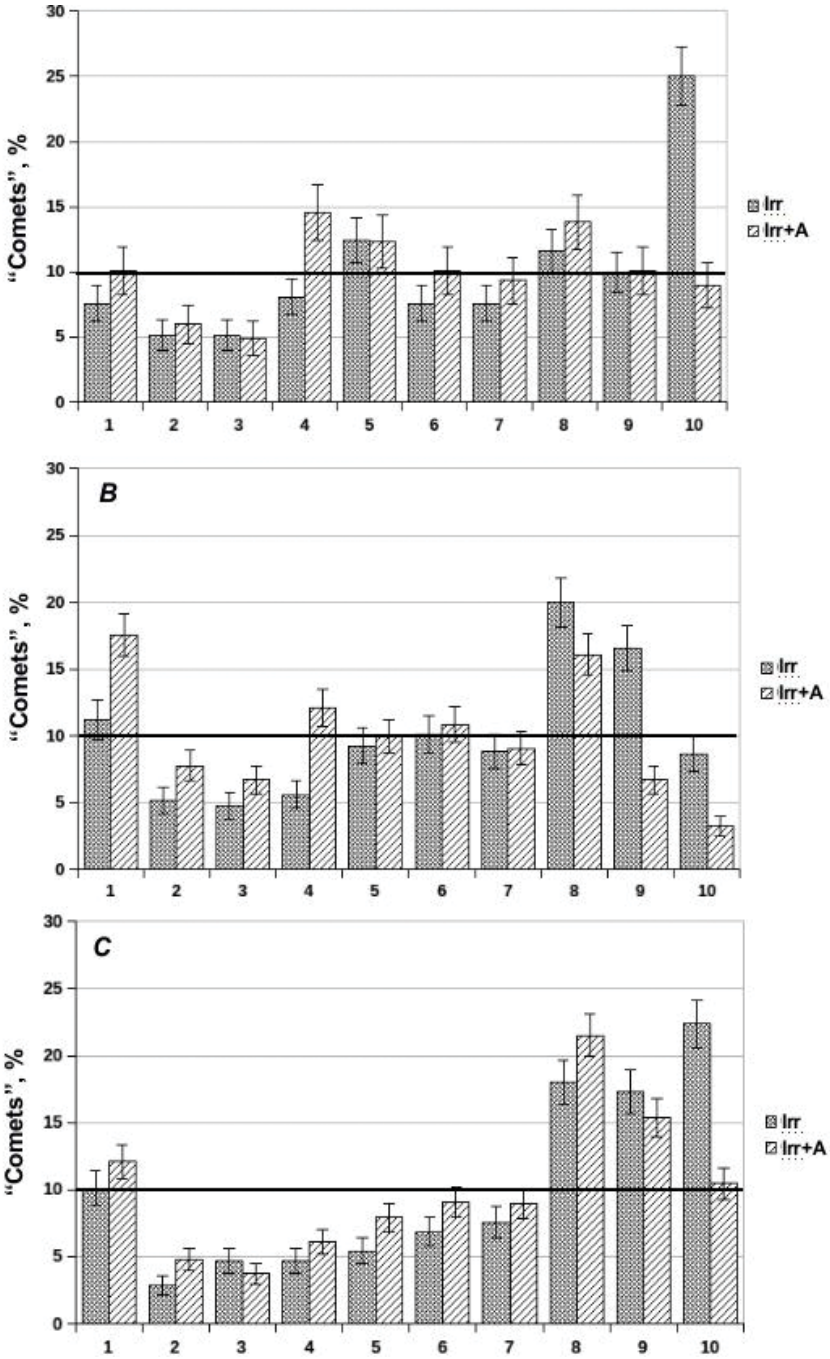

Figure 6. The frequency distribution of "comets" according to the relative levels of DNA damages (see explanation in the text) after irradiation at the 0th hour of cultivation (A), at the 40th hour of cultivation (B), and at the 46th hours of cultivation (C). Irr, irradiated in dose 1.0 Gy cultures; Irr + A, irradiated and supplemented with 20.0 μg/ml astaxanthin cultures. In one to ten groups of "comets," 10% (bold line) is the control value for all groups [28].

with the evaluation of the levels of DNA damages, the count of "atypical comets" (AC) (**Figure 7**) was carried out. It is obvious that AC were formed from the apoptotic cells, because the radiation dose we applied is quite low and cannot induce DNA fragmentation like this [39], while intensive DNA fragmentation occurs exactly during apoptosis [33].

In control cultures after 48 h of cultivation, the AC level was low and did not exceed 1.45 ± 0.53%. The irradiation of lymphocyte cultures at the G_0 stage of the cell cycle with further cultivation led to an increase in the frequency of AC from to 3.11 ± 0.71% ($p < 0.05$), but such effect was not observed after radiation exposure neither at the 40th hour nor at the 46th hours of cultivation (**Figure 7**).

Astaxanthin in concentration of 20 μg/ml per se did not affect the amount of apoptotic cells in non-irradiated cultures of lymphocytes, but the AC level irradiated and treated by astaxanthin cultures was approximately in four times higher than with 48-hour control (7.15 ± 1.13 and 1.69 ± 0.56%, respectively, $p < 0.01$) and in two times higher than with irradiated samples (7.15 ± 1.13 and 3.57 ± 0.81%, respectively, $p < 0.05$). The increase in the frequency of apoptotic cells under the impact of astaxanthin was established exclusively in cultures irradiated at the G_0 stage of the cell cycle and not observed after irradiation in other terms of cultivation.

Thus, similar to cytogenetic effect, the apoptotic activity of astaxanthin was detected only when the irradiated cells were on the G_0 phase of the cell cycle. This may be the cause of the elimination of cells with a large number of DNA breaks and, as consequence, the reduction of the radiation-induced level of chromosomal aberrations we observed earlier.

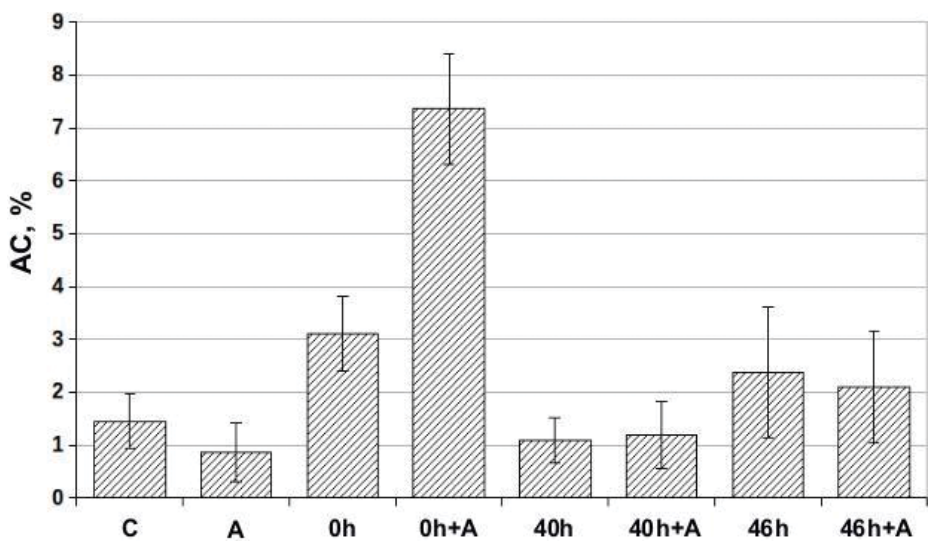

Figure 7. The levels of "atypical comets" (AC%) in cultures of human lymphocytes after 48 hours of cultivation depending on the irradiation terms and the addition of astaxanthin. C, control cultures; A, supplemented with 20.0 μg/ml astaxanthin cultures; 0, 40, and 46 h, cultures irradiated at the 0th, at the 40th, and at the 46th hours of cultivation, respectively; 0 h + A, 40 h + A, and 46 h + A, supplemented with 20.0 μg/ml astaxanthin cultures irradiated at the 0th, at the 40th, and at the 46th hours of cultivation, respectively.

The absence of increase in the frequency of apoptosis after treatment in other terms of cultivation both under the influence of only ionizing radiation and under the combined action of γ-radiation and astaxanthin can be explained by either insufficient time for realization of apoptosis or existence of contingent on the stage of the cell cycle apoptotic pathways, which astaxanthin is unable to activate on the S and G_2 phases of the cell cycle.

Since the increase in the level of apoptosis is not a reason for the decline of the pool of high damaged cells under the astaxanthin influence after irradiation at 40 and 46 h of cultivation, the question remains: what is the cause of such effect of astaxanthin?

It is generally accepted that reactive oxygen species, which are formed by ionizing radiation exposure, cause DNA breaks [40]. Astaxanthin is a power antioxidant and capable to scavenge and quench free radicals and ipso facto to reduce the overall level of DNA damages [3]. According to the data presented in **Figure 7**, the results obtained after irradiation of cells at 46 h of cultivation can be explained by the antioxidant properties of astaxanthin: reducing of oxidative stress leads to a decrease in the number of "comets" of the ten groups and increasing (although not always statistically significant) in the frequencies of cells belonging to other groups (except 3 and 9).

However, such impact of astaxanthin was not observed when cells were irradiated at 40 h of cultivation. It is noteworthy that in this experiment the increase in the frequency of the "comets" of group 1 (from 11.18 ± 1.46 to 17.54 ± 1.62%, $p < 0.01$) was detected. It can be explained by the fact that this group may include cells having a sufficiently large number of lesions enough to trigger mechanisms for the cell cycle arrest on the S phase. Probably, astaxanthin activates S-/G_2-phase checkpoint that leads to an increase in the frequency of the cells from which the "comets" with low DNA are formed (by delay in S phase) and may cause decreasing in the frequency of the "comets" of groups 9 and 10. The results are consistent with the literature data concerning the effects of astaxanthin on the proliferation of tumor cells [7, 17, 41].

4. Conclusion

The obtained results enable us to resume the following astaxanthin effects on irradiated cells that may be clearly observed depending on the phase of the cell cycle and the duration of cells cultivation after irradiation:

1. Stimulation of apoptosis in the irradiated cells resulting in a decrease in the level of cells with a large number of DNA damages (irradiation on the G_0 phase of the cell cycle and cultivation after irradiation for 48 h)
2. Stimulation of the processes that lead to the activation of the checkpoints on the S phase and, accordingly, arrest the division of the most damaged cell population (irradiation on the S phase of the cell cycle and cultivation after irradiation for 8 h)
3. Scavenge of reactive oxygen species resulting in reduction in the total level of DNA breaks (irradiation on the G_2 phase of the cell cycle and cultivation after irradiation for 2 h)

All of these effects are potentially radio- and genoprotective. However, we have previously shown that the protective action of astaxanthin concerning the radiation-induced cytogenetic effect similarly to its apoptotic effect was observed exclusively when irradiated cells were on the G_0 phase of the cell cycle. Moreover, analyzing the ChA spectra (**Figure 3**), we found that supplementation with astaxanthin reduces exactly the levels of classic unstable cytogenetic markers of radiation exposure (dicentric and centric ring chromosomes), and it is known that the cells bearing unstable chromosomal aberrations are eliminated by apoptosis in the first place [42]. So, the radioprotective effect of astaxanthin rather may be due to its ability to stimulate apoptosis in cells that carry a subcritical number of DNA breaks than its potential genoprotective properties (defenses DNA from damages or activates of DNA repair processes).

Author details

Denys Kurinnyi[1]*, Stanislav Rushkovsky[2], Olena Demchenko[1] and Mariya Pilinska[1]

*Address all correspondence to: kurinnyi.d@gmail.com

1 State Institution "National Research Center for Radiation Medicine of the National Academy of Medical Sciences of Ukraine", Kyiv, Ukraine

2 Educational and Research Center "Institute of Biology and Medicine" Taras Shevchenko National University of Kyiv, Kyiv, Ukraine

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Chapter 8

Effects of *Z*-Isomerization on the Bioavailability and Functionality of Carotenoids: A Review

Masaki Honda, Hayato Maeda, Tetsuya Fukaya and Motonobu Goto

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.78309

Abstract

Carotenoids, the most common fat-soluble plant pigments in nature, are beneficial to human health due to their strong antioxidant activities and abilities to prevent various diseases. Carotenoids have many geometrical isomers forms caused by *E*/*Z*-isomerization at arbitrary sites within the multiple conjugated double bonds. Several studies have addressed that the bioavailability as well as the antioxidant, anticancer, and antiatherosclerotic activities of carotenoids varies among the isomers. In addition, those variations differ among carotenoids: *Z*-isomerization resulted in "positive" or "negative" effect for carotenoids bioavailability and functionality, for example, *Z*-isomers of lycopene are more bioavailable than the all-*E*-isomer, whereas the opposite is observed for β-carotene. Thus, to efficiently promote the beneficial effects of carotenoids by ingestion, it is important to have a good understanding of the impact of *E*/*Z*-isomerization on the corresponding functional changes. The objective of this contribution is to review the effects of carotenoid *Z*-isomerization on bioavailability and functionality and describe their differences among carotenoids.

Keywords: lycopene, β-carotene, astaxanthin, *E*/*Z*-isomer, bioavailability, antioxidant activity

1. Introduction

Carotenoids are the most common lipid-soluble pigments responsible for the colors of plants, animals, and microorganisms, and over 1100 different types of carotenoids have been characterized so far [1, 2]. Carotenoids can be divided into the following two groups: (1) carotenes,

which are nonoxygenated molecules such as lycopene and β-carotene; (2) xanthophylls, which are oxygen-containing molecules such as astaxanthin and fucoxanthin (**Figure 1**) [3]. The daily consumption of carotenoid-rich foods would be beneficial for human health because of their high antioxidant, anticancer, and antiatherosclerotic activities [4–6]. Because carotenoids contain numerous conjugated double bonds, many kinds of geometrical isomers are theoretically possible (**Figure 1C**, **E** and **F**). In general, carotenoids in plants occur predominantly in the (all-*E*)-configuration, whereas the *Z*-isomers are present in the human body and processed foods in considerable quantity, for example, over 50% of total lycopene is present as the *Z*-isomers in serum and tissues [7–9]. Data from several studies have shown that the *Z*-isomerization of carotenoids induced changes in important properties, such as the bioavailability, antioxidant activity, and anticancer activity [10–13]. However, these outcomes vary depending on the type of carotenoid: there were cases where the beneficial effects of carotenoids increased or reduced by the Z-isomerization [10–15]. For example, *Z*-isomers of lycopene and astaxanthin have higher bioavailability than the all-*E*-isomers [12, 16], whereas *Z*-isomers of β-carotene have lower bioavailability than the all-*E*-isomers [14]. Furthermore, the results may depend on the evaluation method used. For instance, when the antioxidant activity of β-carotene was evaluated based on oxidation of the low-density lipoprotein (LDL), the all-*E*-isomer showed higher antioxidant activity than the 9*Z*-isomer [17], whereas the 9*Z*-isomer showed higher antioxidant activity when evaluated based on antiperoxidative activity [18]. Moreover, the beneficial effects of carotenoids differ between the *Z*-isomers. For example, when the antioxidant activity of fucoxanthin was evaluated in 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical-scavenging activity assay, the order of activity was 13*Z*-isomer ≈ 13′*Z*-isomer > all-*E*-isomer > 9′*Z*-isomer [19]. The above findings indicate that a good understanding of the effects of *E*/*Z*-isomerization on functional changes is important for increasing the beneficial effects of carotenoid ingestion and for the industrial processing of carotenoids. The objective of this chapter is to highlight the impact of *E*/*Z*-isomerization of carotenoids on their bioavailabilities, antioxidant activities,

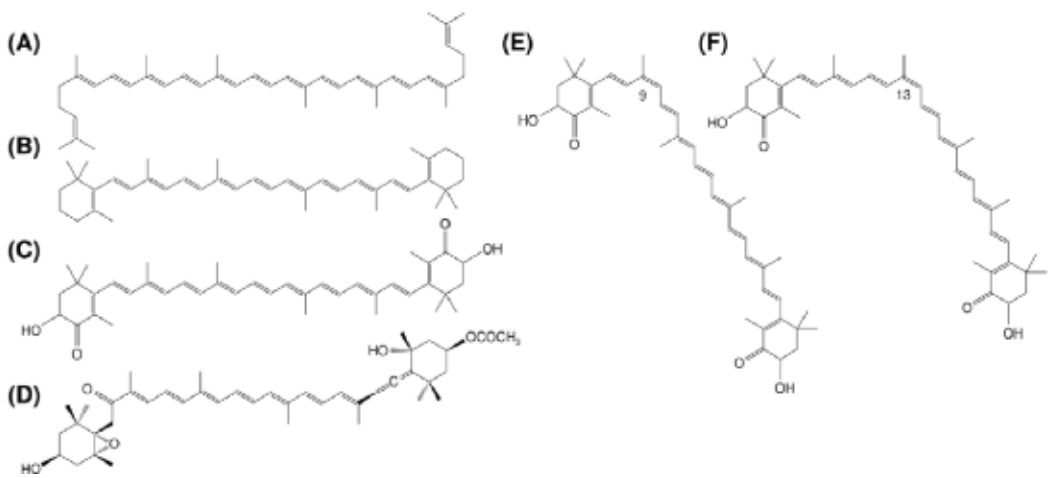

Figure 1. Chemical structures of (A) (all-*E*)-lycopene, (B) (all-*E*)-β-carotene, (C) (all-*E*)-astaxanthin, (D) (all-*E*)-fucoxanthin, (E) (9*Z*)-astaxanthin, and (F) (13*Z*)-astaxanthin.

http://dx.doi.org/10.5772/intechopen.78309

and inhibitory effects against diseases, such as atherogenesis and cancer. Furthermore, aspects of the change factor of the carotenoid bioavailability and functionality, modification of the physicochemical properties of carotenoids by *E*/*Z*-isomerization, and *Z*-isomerization methods used for carotenoids are also discussed in this chapter.

2. Effect of *Z*-isomerization of carotenoids on their bioavailabilities and functionalities

The effects of *Z*-isomerization on the bioavailability and functionality of eight carotenoids have been investigated thus far, including: (1) the bioavailability and antioxidant activity of lycopene; (2) the antioxidant activity of α-carotene; (3) the bioavailability and antioxidant, antiatherogenenic, and antiatherosclerotic activities of β-carotene; (4) the bioavailability and antioxidant activity of astaxanthin; (5) the antioxidant and pro-apoptotic activities of canthaxanthin; (6) the antioxidant and anticancer activities of fucoxanthin; (7) the bioavailability and antioxidant activity of lutein; and (8) the antioxidant activity of zeaxanthin. The changes caused by *Z*-isomerization varied according to the parental carotenoid molecules tested and the evaluation method employed. The findings are described in detail below.

2.1. Lycopene

Lycopene is an acyclic carotene ($C_{40}H_{56}$) that is principally responsible for the bright-red color found abundantly in vegetables and fruits such as tomatoes, guava, and watermelons [3, 9]. Lycopene shows an especially strong antioxidant activity among carotenoids [6] and can significantly reduce the risks for arteriosclerosis, atherogenesis, and many types of cancer (such as prostate and esophageal cancer) [4, 5]. Therefore, in recent years, the use of lycopene in health foods and supplements, and as a natural functional pigment has attracted attention. It is well documented that the bioavailability and antioxidant activity of lycopene are changed by *Z*-isomerization. Most previous findings have demonstrated that the *Z*-isomerization of lycopene results in "positive" health effects.

Data from both *in vitro* and *in vivo* tests have suggested that *Z*-isomers of lycopene are more bioavailable than the all-*E*-isomer. Testing conducted using a diffusion model [20], bile acid micelles [21, 22], human intestinal Caco-2 cells [23], and lymph-cannulated ferrets [21, 22] has provided strong evidence supporting the higher bioavailability of the *Z*-isomers. Moreover, in humans, the ingestion of foods rich in lycopene *Z*-isomers resulted in a measurable increase in blood lycopene concentrations compared to a sample abundant in the (all-*E*)-isomer [12, 24–27]. For example, Cooperstone et al. [12] investigated the effects of ingesting red tomato juice, which mainly contained (all-*E*)-lycopene (90% all-*E*-isomer) and *tangerine* tomato juice, which mainly contained *Z*-isomers of lycopene (94% *Z*-isomers), on plasma lycopene concentrations. Lycopene from the *tangerine* tomato juice showed approximately 8.5-fold greater bioavailability than lycopene from the red tomato juice. Unlu et al. [25] reported that when comparing two tomato sauces—one rich in all-*E*-lycopene (95% all-*E*-isomer) and the other rich in (*Z*)-lycopene (45% *Z*-isomers)—that the *Z*-isomer-rich tomato sauce was approximately

1.5 times more bioavailable than the all-*E*-isomer-rich sauce. In general, the uptake of carotenoids into intestinal mucosal cells is aided by the formation of bile acid micelles [21, 22, 24, 27]. Thus, it is believed that because lycopene *Z*-isomers are more soluble in bile acid micelles than the all-*E*-isomer, they are preferentially incorporated into enterocytes and efficiently form chylomicrons [21, 22]. Indeed, very recently, several reports showed that the solubility of lycopene in oils, organic solvents, and supercritical CO_2 (SC-CO_2) was significantly improved by *Z*-isomerization [28–32]. However, Richelle et al. [33] showed by human oral-dosing tests that the (9*Z*)- and (13*Z*)-isomers were less efficiently absorbed than the 5*Z*- and all-*E* isomers or were converted into 5*Z*- and all-*E* isomers.

Several previous reports have shown that lycopene *Z*-isomers have higher antioxidant activity than the all-*E* isomer and that the relative activities of the isomers varied depending on the assay method [10, 11]. Böhm et al. [10] compared the antioxidant activity of (all-*E*)-lycopene with four unknown *Z*-isomers by measuring their abilities to reduce radical cations of 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (TEAC assay), and each *Z*-isomer showed higher antioxidant activity than the all-*E*-isomer. Müller et al. [11] evaluated the antioxidant activities of (all-*E*)-, (5*Z*)-, (9*Z*)-, (13*Z*)-, and (7*Z*,9*Z*,7′*Z*,9′*Z*)-lycopene using four different *in vitro* assay, namely the TEAC assay, the ferric-reducing antioxidant power (FRAP) assay, the peroxyl radical-scavenging capacity (PSC) assay, and the heme-induced peroxidation of linoleic acid in mildly acidic emulsions mimicking postprandial lipid oxidation in the gastric compartment ($MbFe^{III}$-LP) assay. No significant changes were observed among the isomers in the TEAC and FRAP assay. However, the lycopene *Z*-isomers showed higher antioxidant activities than the all-*E* isomer in the PSC assay (9*Z*-isomer > 5*Z*-isomer ≈ 7*Z*,9*Z*,7′*Z*,9′*Z*-isomer > 13*Z*-isomer > all-*E*-isomer) and in the $MbFe^{III}$-LP assay (5*Z*-isomer > all-*E*-isomer ≈ 9*Z*-isomer ≈ 13*Z*-isomer ≈ 7*Z*,9*Z*,7′*Z*,9′*Z*-isomer). In TEAC assay, Böhm et al. [10] found that *Z*-isomers of lycopene had higher antioxidant activity, but Müller et al. [11] concluded that no significant differences occurred among the isomers. These discrepant findings may be explained by the fact that different concentrations of the isomers were used in each study [10, 11].

Based on the above findings, *Z*-isomerization effectively promotes the beneficial effects of lycopene. Among the *Z*-isomers, (5*Z*)-lycopene would have the highest bioavailability [33] and antioxidant activity [11]. Furthermore, the 5*Z*-isomer has the highest stability of the *Z*-isomers [34–36]. Therefore, regarding lycopene, it is very important to increase the 5*Z*-isomer level and its ingestion. As the *Z*-isomerization method to increase (5*Z*)-lycopene efficiently, heating in some alkyl halides [37] and some kinds of oils such as sesame oil [9, 38], light irradiation with photosensitizers [39], and catalytic treatment [20, 40] were effective. Moreover, to our best knowledge, because the effect of ingesting *Z*-isomer-rich lycopene on inhibiting the development of diseases such as atherogenesis and cancer has not been clarified, further research in that field is expected in the future.

2.2. β-Carotene

β-carotene is a cyclic carotene ($C_{40}H_{56}$) that is found abundantly in vegetables and fruits, and provides vegetables such as carrots and pumpkins with a deep orange-yellow color [3, 4]. As with other carotenoids, β-carotene has a high antioxidant capacity [6] and preventive effect

against various diseases such as cancer and atherogenesis [4, 17]. Furthermore, β-carotene is very important as a retinol precursor, with a high conversion rate [3, 4]. It is also well documented that the bioavailability and antioxidant activity of β-carotene as well as its antiatherogenic activity are changed by *Z*-isomerization. Most previous studies have shown that the *Z*-isomerization results in "negative" effect for bioavailability. In contrast, β-carotene *Z*-isomerization has shown both "positive" and "negative" effects on antioxidant activity, depending on the evaluation method, and "positive" effects have been shown in terms of antiatherogenic activity.

Data from several *in vitro* and *in vivo* tests have indicated that *Z*-isomers of β-carotene are less bioavailable than the all-*E*-isomer. For example, *in vivo* tests using Caco-2 cells, HSC-T6 cells, and liver microsomes [14], as well as *in vivo* tests using ferrets [41] and gerbils [42] have shown this phenomenon. In humans, the intake of *Dunaliella salina* and *Dunaliella bardawil* rich in (9*Z*)-β-carotene showed lower β-carotene bioavailability than foods rich in the all-*E*-isomer [43–48]. The effects of the *Z*-isomer content on the bioavailability were opposite between lycopene and β-carotene. Generally, after carotenoids are extracted from the food matrix and incorporated into mixed micelles, bioaccessible carotenoids can be internalized by enterocytes [21, 22, 24, 27]. The main absorption site of carotenoids is in the duodenum, and several proteins that are temporarily present at the apical membrane mediate selectivity in terms of carotenoid uptake [27, 49–51]. *In vitro* experiments with Caco-2 cells showed that carotenoid transport decreased in the following order: β-carotene ≈ α-carotene (50% inhibition) > β-cryptoxanthin ≈ lycopene (20% inhibition) > lutein: zeaxanthin (1:1) (7% inhibition) [49]. Because carotenoid *Z*-isomers have higher solubility than the all-*E*-isomers [28–32], they can incorporate into bile acid micelles more efficiency [21, 22]. Therefore, it is considered that *Z*-isomers of β-carotene have lower transport efficiency in Caco-2 cell than the all-*E*-isomers [13]. However, a few studies have suggested that *Z*-isomers of β-carotene have higher bioavailability than the all-*E*-isomers, as evaluated using human intestinal Caco-2 cells [52] and ferrets [53]. The use of different delivery systems with the cell model system and animal species might have caused discordant results [15].

Several studies have been conducted to compare the antioxidant activities of (all-*E*)-β-carotene and the *Z*-isomers, and the degree of antioxidant activity detected varied according to the assay method. Namely, the 9*Z*-isomer showed higher antioxidant activity than the all-*E*-isomer when evaluated in terms of the sensitivity to external oxidants [54], the antiperoxidative activity [18], and oral dose testing in rats [55]. However, the opposite results (or no significant differences) were observed when the antioxidant activities were evaluated by measuring the oxidation of LDL [17] or in TEAC assay [10, 56], and PSC assay [56, 57]. Rodrigues et al. [57] reported that β-carotene *Z*-isomers were less efficient as peroxyl radical scavengers than the corresponding all-*E*-isomers: the *Z*-isomers presented the values about 20% lower than that found for the all-*E*-isomer, and they addressed that the negative effect may be due to the decreasing of the orbital overlap. Based on the above findings, it is difficult to conclude whether antioxidant activity is enhanced by *Z*-isomerization of the all-*E*-isomer.

Moreover, as additional "positive" effects of β-carotene *Z*-isomers, it has been reported that the 9*Z*-isomer has higher antiatherogenic activity [58] and antiatherosclerotic activity

[59, 60] than the all-*E*-isomer. On the other hand, there are other "negative" effects. Namely, β-carotene is a very important retinol precursor with a high conversion rate. The (all-*E*)- and (9*Z*)-β-carotene can be metabolized respectively to (all-*E*)-retinoic acid and (9*Z*)-retinoic acid [61, 62], both of which are active in gene regulation [63, 64]. However, the rates of cleavage of β-carotene isomers to vitamin A and the composition of the respective isomer metabolites vary, that is, (all-*E*)-β-carotene was the preferred substrate for cleavage to vitamin A when compared with the *Z*-isomers [61, 65, 66].

Regarding β-carotene, considering that "positive" and "negative" effects are associated with *Z*-isomerization, it is considered important to use them properly depending on the situation. Besides, as the *Z*-isomerization method for (all-*E*)-β-carotene, heating [67, 68], light irradiation with photosensitizers [69], and catalytic treatment [70, 71] were well documented. Moreover, *Dunaliella salina* and *Dunaliella bardawil*, which contain a large amount of (9*Z*)-β-carotene, have been used as *Z*-isomer-rich materials [43–48].

2.3. Astaxanthin

Astaxanthin is a xanthophyll ($C_{40}H_{52}O_4$) that is principally responsible for the dark-red color in various microalgae and marine animals [1, 72]. Astaxanthin shows an especially strong antioxidant activity among carotenoids [6] and can significantly reduce the risk of cancer, eye disease, and cardiovascular disease [73, 74]. For instance, astaxanthin protected mice from carcinogenesis of the urinary bladder by reducing the incidence of chemically induced bladder carcinoma and further, astaxanthin supplementation in rats inhibited the stress-induced suppression of tumor-fighting natural killer cells [73]. In addition, astaxanthin is frequently used as an animal and fish feed additive to improve their body colors [75]. Data from several studies have demonstrated that the bioavailability and antioxidant activity of astaxanthin were changed by *Z*-isomerization.

In terms of the bioavailability, an *in vitro* test using a simulated digestion model and human intestinal Caco-2 cells [76] and human oral-dosing studies [16, 77] have shown that *Z*-isomers have higher bioavailability than the all-*E*-isomer. For example, Yang et al. [76] reported that (13*Z*)-astaxanthin showed higher bioaccessibility than (9*Z*)- and (all-*E*)-astaxanthins using an *in vitro*-digestion model, and (9*Z*)-astaxanthin exhibited higher cellular-transport efficiency than (all-*E*)- and (13*Z*)-astaxanthin in Caco-2 cell monolayers. However, oral-dosing studies in rainbow trout (*Oncorhynchus mykiss*) have shown a "negative" effect of astaxanthin *Z*-isomerization on bioavailability [78, 79]. These results suggest that the bioavailability of carotenoid isomers differs among species. Thus, future studies should seek to establish the biochemical basis for species-specific differences in the utilization of carotenoid isomers.

Although the antioxidant activity measured depends on the assay method employed, many studies have shown "positive" effects. Namely, assay that measure antioxidant enzyme activities, DPPH radical scavenging, oxygen radical-absorption capacity (ORAC), photochemiluminescence (PLC) and peroxidation have shown higher antioxidant activities of astaxanthin *Z*-isomers than detected for the all-*E*-isomer [76, 80, 81]. In contrast, when the antioxidant activity was evaluated by a cellular antioxidant activity (CAA) assay, the order of

the antioxidant activity was 13*Z*-isomer > all-*E*-isomer > 9*Z*-isomer [81]. The results of these studies suggest that *Z*-isomers of astaxanthin, especially the 13*Z*-isomer, have higher antioxidant activity than the all-*E*-isomer.

Most investigators have concluded that "positive" effects on the bioavailability and antioxidant activity occurred following astaxanthin *Z*-isomerization. Thus, the ingestion of astaxanthin *Z*-isomers could be effective in these terms. As with other carotenoids, *Z*-isomers of astaxanthin could be obtained by heating [81, 82] and catalytic treatment [76, 81, 83] of the all-*E*-isomer.

2.4. Canthaxanthin

Canthaxanthin is a xanthophyll ($C_{40}H_{52}O_2$) that is principally responsible for the orange-pink color found abundantly in egg yolk and various microbes such as *Bradyrhizobium* sp. and *Halobacterium* sp. [84, 85]. Canthaxanthin can significantly reduce the risk of cancer and neurodegenerative disorder [86, 87] and shows strong antioxidant activity [88], that is, canthaxanthin administration decreased mammary tumor volumes in mice [86] and exhibited antiinflammatory activities by increasing the activity of GPX and catalase, thereby reducing the production of IL-1, IL-6, and TNF-α [87]. Furthermore, canthaxanthin is widely used as feed for hens and fish to improve the egg yolk color and the body color, respectively [84, 89]. A few reports have shown the effect of canthaxanthin *Z*-isomerization on antioxidant activity and functionality. Venugopalan et al. [88] reported that (9*Z*)-canthaxanthin isolated from *Dietzia* sp. had higher antioxidant activity, as evaluated by performing DPPH radical-scavenging assay, superoxide radical-scavenging assay and fluorescence assay to detect reactive oxygen species generated in THP-1 cells. Moreover, the (9*Z*)-isomer exhibited higher pro-apoptotic activity than the all-*E*-isomer, which was evaluated in THP-1 macrophages [90]. The above literature indicates that *Z*-isomerization of canthaxanthin has "positive" effects. Canthaxanthin *Z*-isomerization can be achieved by heating and catalytic treatment [91, 92], and *Dietzia* sp. can serve as a source of (9*Z*)-canthaxanthin [88, 89].

2.5. Fucoxanthin

Fucoxanthin is an allenic xanthophyll ($C_{42}H_{58}O_6$) that is found abundantly in edible shellfish and brown seaweeds such as *Mactra chinensis* and *Undaria pinnatifida* [1, 93]. Fucoxanthin has high antioxidant capacity [94] and shows anticancer and antiangiogenic activities [95, 96]. For example, fucoxanthin remarkably reduced the viability of human colon cancer cell lines, such as Caco-2, HT-29, and DLD-1 cells [95]. In addition, fucoxanthin has antiobesity and antidiabetic effects [97–99], for example, administration of Wakame (*Undaria pinnatifida*) (which is rich in fucoxanthin) significantly suppressed body weight and white adipose tissue weight gain induced by the high fat diet in an obese murine model [98], which has attracted much attention recently in the food industry. The *Z*-isomerization of (all-*E*)-fucoxanthin can induce changes in the antioxidant and anticancer activities. Namely, Zhang et al. [19] reported that when the antioxidant activity of fucoxanthin isomers was evaluated by performing DPPH radical-scavenging and superoxide-detection assay, the following relative activities were observed: 13*Z*-isomer ≈ 13′*Z*-isomer > all-*E*-isomer > 9′*Z*-isomer. Evaluation by performing

2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and hydroxyl radical-scavenging assay revealed the following relative activities: 9′*Z*-isomer > all-*E*-isomer >13*Z*-isomer ≈ 13′*Z*-isomer. Moreover, Kawee-ai et al. [100] showed that when the ratio of the *Z*-isomer of fucoxanthin increased by 2% from 11 to 13% by heating, the scavenging activities against DPPH, hydrogen peroxide, and superoxide anions, and the reducing power decreased by 21.0, 10.3, 16.0 and 19.7%, respectively. Hence, it is considered that the *Z*-isomerization of fucoxanthin negatively affects the antioxidant activity. However, Nakazawa et al. [13] demonstrated that *Z*-isomers of fucoxanthin had higher anticancer activity than the all-*E*-isomer, as evaluated by measuring the potent inhibitory effects on human promyelocytic leukemia cells (HL-60) and colon cancer cells (Caco-2). To the best of our knowledge, only fucoxanthin was investigated in terms of the effect of *Z*-isomerization on anticancer activity. Thus, it is expected that such investigation will extend to other carotenoids in the future. *Z*-isomerization of (all-*E*)-fucoxanthin has been achieved by heating and light irradiation [100, 101].

2.6. Lutein

Lutein is a xanthophyll ($C_{40}H_{56}O_2$) that is principally responsible for the yellow-orange color found abundantly in vegetables, for example, corn, carrots, kale, and peas, and in egg yolks [102]. Lutein has preventive effects against various diseases such as eye diseases and cardiovascular diseases [102–104]. In particular, several studies have addressed the role of lutein in reducing the risk of the two most common eye diseases in older people, that is, cataracts and macular degeneration [102–104]. Only a few reports have shown the effect of lutein *Z*-isomerization on bioavailability and antioxidant activity [15, 105]. *In vitro* tests using a digestion model have shown a higher bioaccessibility of *Z*-isomers of lutein than the all-*E*-isomer, and a Caco-2 cell monolayer model has shown a lower bioavailability. These results indicated that *Z*-isomers of lutein are more efficiently incorporated into bile acid micelles, but they have lower transport efficiency in enterocytes via the activities of carotenoid-transport proteins like β-carotene, as described above [15, 27, 49–51].

In terms of antioxidant activity, the *Z*-isomers, especially the 13′*Z*-isomer, have shown higher antioxidant activities than the all-*E*-isomer in FRAP, DPPH, and ORAC assay, but no significant differences in the activities of the isomers were observed in CAA assay [15]. Since few reports are available regarding the effects of *Z*-isomerization on lutein bioavailability and functionality, and no such studies have been conducted in humans, further studies are needed to clarify whether *Z*-isomerization shows "positive" or "negative" effects. Several studies have reported that (all-*E*)-lutein can be isomerized to the *Z*-isomers by heating [106, 107] and catalytic treatment [15].

2.7. Other carotenoids

The effects of *Z*-isomerization on the antioxidant activities of other carotenoids, such as α-carotene and zeaxanthin, were investigated by Böhm et al. [10] by performing TEAC assay. The following relative antioxidant activities of α-carotene stereoisomers were found: 13′*Z*-isomer > all-*E*-isomer ≈ 9′*Z*-isomer > 9*Z*-isomer ≈ 13*Z*-isomer, whereas those for zeaxanthin were as follows: all-*E*-isomer ≈ 13*Z*-isomer > 9*Z*-isomer. It is difficult to discern whether

Carotenoid	Evaluation	Overview of results	Effect*	Reference
Lycopene	Bioavailability/ bioaccessibility	*Z*-Isomers > all-*E*-isomer, evaluated using a diffusion model	+	[20]
		Z-Isomers > all-*E*-isomer, evaluated using bile acid micelles and lymph-cannulated ferrets	+	[21, 22]
		Z-Isomers > all-*E*-isomer, evaluated in Caco-2 cells	+	[23]
		Z-Isomers > all-*E*-isomer, evaluated in human oral-dosing tests	+	[12, 24–26]
	Antioxidant activity	*Z*-Isomers > all-*E*-isomer, evaluated in TEAC assay	+	[10]
		Z-Isomers > all-*E*-isomer, evaluated in PSC and $MbFe^{III}$-LP assay	+	[11]
		All-*E*-isomer ≈ *Z*-isomers, evaluated in TEAC and FRAP assay	±	[11]
α-Carotene	Antioxidant activity	13′*Z*-Isomer > all-*E*-isomer ≈ 9′*Z*-isomer > 9*Z*-isomer ≈ 13*Z*-isomer, evaluated in TEAC assay	±	[10]
β-Carotene	Bioavailability/ bioaccessibility	All-*E*-isomer > *Z*-isomers, evaluated in Caco-2 cells, HSC-T6 cells, and rat liver microsomes	−	[14]
		All-*E*-isomer > *Z*-isomers, evaluated in ferret oral-dosing test	−	[41]
		All-*E*-isomer > *Z*-isomers, evaluated in gerbil oral-dosing test	−	[42]
		All-*E*-isomer > 9*Z*-isomer, evaluated in human oral-dosing tests	−	[43–48]
		9*Z*-Isomer > all-*E*-isomer, evaluated in Caco-2 cells	+	[52]
		9*Z*-Isomer > all-*E*-isomer, evaluated in the small intestines of ferrets	+	[53]
	Antioxidant activity	9*Z*-Isomer > all-*E*-isomer, evaluated by measuring the sensitivity to external oxidants	+	[54]
		9*Z*-Isomer > all-*E*-isomer, evaluated by determining the antiperoxidative activity	+	18
		9*Z*-Isomer > all-*E*-isomer, evaluated in rat oral-dosing tests	+	[55]
		All-*E*-isomer > 9*Z*-isomer, evaluated by measuring LDL oxidation	−	[17]
		All-*E*-isomer ≈ 9*Z*-isomer ≈ 13*Z*-isomer ≈ 15*Z*-isomer, evaluated in TEAC assay	−	[10]
		All-*E*-isomer ≈ 9*Z*-isomer ≈ 13*Z*-isomer > 15*Z*-isomer, evaluated in TEAC and PSC assay	−	[56,57]
	Atherogenesis activity	9*Z*-Isomer > all-*E*-isomer, evaluated in knockout mice	+	[58]
	Atherosclerosis activity	9*Z*-Isomer > all-*E*-isomer, evaluated in female LDLR−/− and apoE-deficient mice	+	[59, 60]

Carotenoid	Evaluation	Overview of results	Effect*	Reference
Astaxanthin	Bioavailability/ bioaccessibility	*Z*-Isomers > all-*E*-isomer, evaluated using a digestion model and Caco-2 cells	+	[76]
		13*Z*-Isomer > all-*E*-isomer, 9*Z*-isomer, evaluated in human oral-dosing test	+	[77]
		Z-Isomers > all-*E*-isomer, evaluated in human oral-dosing test	+	[16]
		All-*E*-isomer > *Z*-isomers, evaluated in rainbow trout (*Oncorhynchus mykiss*) oral-dosing tests	−	[78, 79]
	Antioxidant activity	*Z*-Isomers > all-*E*-isomer, evaluated in antioxidant enzyme-activity assay	+	[76]
		Z-Isomers > all-*E*-isomer, evaluated in DPPH and lipid-peroxidation assay	+	[80]
		Z-Isomers > all-*E*-isomer, evaluated in DPPH, ORAC, and PLC assay	+	[81]
		13*Z*-Isomer > all-*E*-isomer >9*Z*-isomer, evaluated in CAA assay	±	[81]
Canthaxanthin	Antioxidant activity	9*Z*-Isomer > all-*E*-isomer, evaluated in DPPH, superoxide radical-scavenging, and fluorescence assay	+	[88]
	Pro-apoptotic activity	9*Z*-Isomer > all-*E*-isomer, evaluated in THP-1 macrophages	+	[90]
Fucoxanthin	Antioxidant activity	13*Z*-Isomer ≈ 13′*Z*-isomer > all-*E*-isomer > 9′*Z*-isomer, evaluated in DPPH and superoxide-detection assay	±	[19]
		9′*Z*-Isomer > all-*E*-isomer > 13*Z*-isomer ≈ 13′*Z*-isomer, evaluated in ABTS and hydroxyl radical-scavenging assay	±	[19]
		Z-Isomers > all-*E*-isomer, evaluated in DPPH, hydrogen peroxide-scavenging, superoxide anion, and reducing-power assay	−	[100]
	Anticancer activity	*Z*-Isomers > all-*E*-isomer, evaluated in HL-60 cells and Caco-2 cells	+	[13]
Lutein	Bioavailability/ bioaccessibility	*Z*-Isomers > all-*E*-isomer, evaluated using a digestion model	+	[15]
		All-*E*-isomer > *Z*-isomers, evaluated in Caco-2 cells	−	[15]
		13*Z*-Isomer > all-*E*-isomer, evaluated using a digestion model	+	[105]
	Antioxidant activity	*Z*-Isomers > all-*E*-isomer, evaluated in FRAP assay	+	[15]
		13′*Z*-Isomer > all-*E*-isomer ≈ 9*Z*-isomer, evaluated in DPPH and ORAC assay	+	[15]
		All-*E*-isomer ≈ *Z*-isomers, evaluated in CAA assay	±	[15]
Zeaxanthin	Antioxidant activity	All-*E*-isomer ≈ 13*Z*-isomer > 9*Z*-isomers, evaluated in TEAC assay	−	[10]

*Expected effect of carotenoid *Z*-isomerization on humans: +, "positive" effect; −, "negative" effect; ±, no change or indetermine.

Table 1. Summary of the effects of ***Z***-isomerization of different carotenoids on the bioavailability and functionality.

Z-isomers of both carotenoids have higher antioxidant activity than the all-*E*-isomer based on the TEAC assay results alone; thus, further evaluations by multiple testing methods are necessary.

To the best of our knowledge, the effect of *Z*-isomerization of other important carotenoids such as capsanthin and β-cryptoxanthin (which have large markets and high functionalities) on the bioavailability and functionality has not been reported. Among the over 1100 reported carotenoids found in nature, only the eight carotenoids mentioned above have been characterized in terms of the effects of *Z*-isomerization, as summarized in **Table 1**. Thus, further progress in this research area is expected in the future.

3. Changes in the physicochemical properties of carotenoids by *Z*-isomerization

Changes in the bioavailability and functionality of carotenoids after *Z*-isomerization should have strong correlations with changes in their physicochemical properties. Several reports have shown that the *Z*-isomerization of carotenoids can induce changes in various properties such as the stability, solubility, and crystallinity. Some computational approaches using a Gaussian program have revealed that the *Z*-isomerization of carotenoids affected the Gibbs free energy [34, 35, 108, 109], that is, the relative stability of all-*E*- and mono-*Z*-isomers were in the following order: all-*E*-isomer ≈ 5*Z*-isomer > 9*Z*-isomer > 13*Z*-isomer > 15*Z*-isomer > 7*Z*-isomer ≈ 11*Z*-isomer for lycopene [34, 35, 108]; all-*E*-isomer > 9*Z*-isomer > 13*Z*-isomer > 15*Z*-isomer > 7*Z*-isomer ≈ 11*Z*-isome for β-carotene [109]. Thus, (all-*E*)-carotenoids should be more stable than the *Z*-isomers, which was confirmed experimentally by Murakami et al. [36] using lycopene isomers. Changes in the Gibbs free energy, stability, of carotenoids following *Z*-isomerization would affect their antioxidant activities. In addition, there is limited experimental evidence that the *Z*-isomers of carotenoids such as lycopene, β-carotene, and astaxanthin have higher solubility in vegetable oil, organic solvents and SC-CO_2 than the all-*E*-isomer [28–32, 110, 111], for example, the solubility of lycopene *Z*-isomers in ethanol was over 4000 times higher than that of the all-*E*-isomer [29]. These properties should affect the bioavailability of carotenoids. Namely, *Z*-isomerization of carotenoids could enhance uptake into bile acid micelles due to an increased solubility; thus, the bioavailability of lycopene and astaxanthin was improved [20–22]. On the other hand, regarding β-carotene and lutein, whose *Z*-isomers showed lower bioavailability [15, 41–48, 105], the uptake into bile acid micelles could potentially be improved by *Z*-isomerization, but they might have lower transport efficiency in enterocytes due to the activities of several carotenoids transport proteins, which are temporarily present at the apical membrane [27, 49–51]. *In vitro* tests of lutein support this hypothesis, that is, the *Z*-isomers showed higher bioaccessibility than the all-*E*-isomers in a digestion model [15, 105], whereas the opposite result was obtained in Caco-2 cells [15]. It has been predicted that *Z*-isomers of lycopene and astaxanthin can be efficiently internalized via carotenoid transporters, based on the results of testing conducted using Caco-2 cells [23, 76]. The abovementioned theory is strongly supported by the observations that, in human blood, over 50% of total lycopene exists in the *Z*-form, but only 5% of total β-carotene exists in the *Z*-form [112]. To attain a better understanding of the underlying mechanisms, further study

on the uptake process of (*Z*)-carotenoids in enterocytes by carotenoid transport proteins is necessary. Furthermore, the crystallinity of carotenoids was changed by *Z*-isomerization: although (all-*E*)-carotenoids existed in a crystalline state, the *Z*-isomers were in an amorphous state, which was confirmed by optical observations, differential scanning calorimetry, powder X-ray diffraction, and scanning electron microscopy analyses [20, 28, 29, 113]. The change in crystallinity resulting from *Z*-isomerization may also influence changes in carotenoid bioavailability and functionality.

4. Conclusions

Z-Isomerization of carotenoids can cause changes in the bioavailability, antioxidant activity, and other functionalities (such as anticancer and antiatherogenic activities), and it may result in "positive" or "negative" effects, which vary according to the type of carotenoid. Although more than 1100 carotenoids are found in nature, only the eight carotenoids discussed above have been investigated in terms of these effects. Thus, further progress in this research area is expected. Furthermore, most investigations have focused on the effects of *Z*-isomerization of carotenoids on the bioavailability and antioxidant activity, but the *Z*-isomerization has been shown to enhance the anticancer and antiatherogenic activities of β-carotene and fucoxanthin. Since these data provide important evidence for the roles of carotenoid *Z*-isomerization in human health, examination of other carotenoids is expected in the future.

Author details

Masaki Honda[1]*, Hayato Maeda[2], Tetsuya Fukaya[3] and Motonobu Goto[4]

*Address all correspondence to: honda@meijo-u.ac.jp

1 Faculty of Science and Technology, Meijo University, Nagoya, Japan

2 Faculty of Agriculture and Life Science, Hirosaki University, Aomori, Japan

3 Innovation Division, Kagome Company, Limited, Nishitomiyama, Nasushiobara, Japan

4 Department of Materials Process Engineering, Nagoya University, Nagoya, Japan

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Chapter 9

Lutein-Enriched Emulsion-Based Delivery System: Impact of Casein-Phospholipid Emulsifiers on Chemical Stability

Adela Mora-Gutierrez, Rahmat Attaie and Maryuri Núñez de González

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.78601

Abstract

The health benefits of carotenoids in terms of their role in decreasing the risk of diseases, particularly certain cancers and eye disease, are limited by their chemical degradation. Emulsion delivery systems with water dispersions of a carotenoid enhance chemical stability and bioavailability to the host. An emulsified carotenoid delivery system can be based on carotenoid dissolution in lipid media and its stabilization by a surfactant mixture of milk proteins (the caseins) and phospholipids. The inclusion of lutein into an emulsified delivery system comprised of bovine casein or caprine casein in combination with phospholipids (soybean lecithin) enhanced the chemical stability of lutein during storage for 7 days at pH 7.0 at incubation temperatures of 5 and 15°C. The chemical stability of lutein in the corn oil-in-water emulsions stabilized by bovine and caprine caseins in combination with soybean lecithin was in the following order: caprine α_{s1}-II-casein/lecithin > caprine α_{s1}-I-casein/lecithin > bovine casein/lecithin. The results suggest that the chemical stability of lutein in oil-in-water emulsions can be enhanced by altering the thickness of the interfacial layer. Caprine casein/lecithin has the potential for use as an emulsifier in beverage emulsions.

Keywords: lutein, casein, phospholipids, emulsion, stability

1. Introduction

Among the polar oxygenated xanthophylls of the carotenoids, lutein has received attention for its potent antioxidant activity [1]. Lutein may protect the DNA of photoreceptive cells in

the retina from the harmful effects of strong light [2]. In the skin, lutein is believed to protect against UV radiation [3]. Lutein is naturally synthesized by plants, and is commercially available as a food supplement from Marigold flowers (*Tagetes erecta*) [4]. Another source of lutein, and one that is the most bioavailable of all, is the egg. A large egg yolk contains approximately 252 μg of lutein (and zeaxanthin); while there is not a tremendous amount of lutein in egg yolks, it is so bioavailable that it is taken into the bloodstream with great efficiency, giving a significant boost to the serum levels of this protective carotenoid [5]. Indeed, the inclusion of lutein in a lipophilic matrix (egg yolk phospholipids) leads to dramatic improvement in the absorption of lutein in humans [6]. However, the segment of the population that has been recommended to control dietary cholesterol intake avoids consumption of eggs. American adults have a poor supply of lutein and their intake of ~1–2 mg/day by eating fruits and vegetables may not be enough to attain health benefits [7].

Fortification of foods and beverages with fat-soluble bioactive components such as the carotenoids, especially β-carotene, is considered important in the elimination of acute deficiency symptoms, in optimizing health, and in providing protection from many chronic ailments on a long-term basis [8, 9]. The difficulties encountered in fortifying foods with the carotenoids are primarily due to their instability (air, light) and low water solubility [10]. Since carotenoids cannot be incorporated into aqueous-based foods, an emulsion-based delivery system provides a suitable means for dispersing the lipophilic carotenoids into the aqueous environments of foods [11]. Emulsion-based delivery systems also allow for improved absorption of non-polar or fat-soluble bioactive compounds such as the carotenoids [12]. The type of emulsion used should be considered when carotenoids are added to food and beverage products to inhibit the rate of their degradation [11].

Food emulsions are usually one of the simplest forms of oil-in-water emulsions, consisting of small oil droplets dispersed within an aqueous medium, with the oil droplets having mean diameters ranging from 10 to 100 nm (nanoemulsions) or 100 nm to 100 μm (conventional emulsions). The formation of successful emulsion-based food and beverage products requires emulsifiers [13]. Emulsifiers are surface-active (surfactants) substances that play a crucial role in the mixing of two immiscible liquids. These surface active substances normally have a polar (hydrophilic) and a non-polar (hydrophobic) ends that break the surface tension between different liquids thereby, facilitating the formation of the mix and maintaining the stability of the mix. Food manufacturers have traditionally used both synthetic and natural emulsifiers in food formulations; however, the clean label movement is creating a new trend toward the use of natural emulsifiers. In this context, the interest of the general public and the food industry professionals are toward identifying, characterizing, and utilizing naturally occurring substances such as proteins, polysaccharides, phospholipids, and saponins as emulsifiers in formulated foods. The desire is to find which naturally occurring compounds have the appropriate properties to efficiently form stabilized emulsions in foods with possible commercial applications.

Proteins contain a mixture of hydrophilic and hydrophobic amino acids along their polypeptide chains that render them naturally surface active agents. This characteristic enables most proteins to quickly absorb to oil-in-water interfaces and coat the oil droplets that are formed during mixing and homogenization. The negatively charged carboxylic groups ($–COO^-$) or the positively charged amino groups ($–NH_3^+$) of amino acids that make up proteins can stabilize

droplets from aggregation by generating electrostatic repulsions. Proteins are generally relatively small molecules (about 10–50 kDa) that rapidly absorb to droplet surfaces and form thin, electrostatically charged interfacial layers. Such layers are important in the formation of stabilized emulsions.

Currently, caseins and whey proteins from bovine origin are the most commonly used protein-based emulsifiers in the food industry. Caseins are amphiphilic proteins with flexible structures (α_{s1}-, α_{s2}-, β-, and κ-caseins) whereas whey proteins are globular proteins with fairly rigid structures [β-lactoglobulin, bovine serum albumin (BSA), and immunoglobulins]. Due to their structural flexibility, caseins rapidly undergo conformational changes, with the hydrophilic groups protruding into the water phase and the hydrophobic groups into oil phase.

Phospholipids, similar to proteins, are amphiphilic molecules (loving both water and oil) with hydrophobic fatty acid tail groups and phosphoric acid esterified with glycerol and other substitutes as the hydrophilic head groups. Phospholipids are generally referred to as lecithin, and they occur in nature in the cell membranes of animals, plants, and microbial species. Phospholipids are industrially extracted from soybeans, egg yolk, milk, and sunflower kernels for use in foods. Lecithin contains a mixture of different phospholipids, with the most common being phosphatidylcholine, phosphatidylethanolamine, and phosphatidylinositol. While lecithin ingredients are surface-active agents and facilitate the mixing of oil and water, they also are prone to coalescence because they form interfacial layers. Combining lecithin with proteins such as bovine caseins [14] can minimize this issue and help form stable emulsions.

Milks from different mammalian species present differences in their relative proportions and characteristics of caseins [15]. The degree of variability of caprine casein components from the individual milks of French-Alpine and Anglo-Nubian breeds of goats has been previously reported [16]. For both breeds the quantity of β- and κ-caseins are relatively constant while the content of α_s-caseins of these breeds vary significantly [16]. In bovine milk the constancy of the ratio of caseins results in a rather distribution of structure for casein micelles (with Ca^{2+}), which has been used for the delivery of fat-soluble bioactive compounds such as curcumin and vitamin D in aqueous solutions [17, 18]. Casein sub-micelles (without Ca^{2+}) from bovine and caprine milks have been used for the delivery of lutein in food emulsions [19, 20].

The aim of our study was to evaluate the effects of bovine and caprine caseins (sub-micelles) in combination with phospholipids (soy lecithin) as emulsifiers on the chemical stability of lutein in corn oil-in-water emulsions at pH 7.0 during storage at 5 and 15°C. The corn oil allows the carotenoids to be absorbed [21].

2. Materials and methods

2.1. Materials

A commercial preparation of lutein consisting of 20% (wt/wt) lutein dissolved in corn oil was a gift from Hoffman La Roche (Pleasanton, CA). Mazola corn oil was purchased from a local supermarket. A lutein standard for chromatographic analysis was purchased from

Extrasynthèse SA (Genay, France). Soybean lecithin Beakin LV3 was a gift from Archer Daniels Midland Co (Decatur, IL, USA). Ethanol, thimerosal, phenylmethanesulfonyl fluoride, and monobasic potassium phosphate were purchased from Sigma-Aldrich (St. Louis, MO, USA). Dimethyl sulfoxide (DMSO) was purchased from Fisher Scientific (Pittsburg, PA, USA). Deionized water was prepared by passing distilled water over a mixed bed of cation-anion exchanger and was used throughout this study.

2.2. Source of caseins

Whole caseins were prepared by isoelectric precipitation of the individual skimmed milk samples obtained from a Jersey cow and French-Alpine goats. The precipitate was dissolved by the addition of NaOH to yield a solution of pH 7.0. The casein was re-precipitated, washed, and then re-suspended. The sodium caseinate was subsequently cooled to 4°C and centrifuged at 100,000 × g for 30 min to remove residual fat using a Beckman Optima XL-A (Beckman Instruments Inc., Palo Alto, CA, USA) analytical ultracentrifuge. Finally, the suspension was dialyzed against cold deionized water at 4°C for 72 h with three changes and then lyophilized. The integrity of the samples was confirmed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and the percentages of the various component caseins estimated by densitometry as previously described [22]. The casein composition of the samples is as follows: α_{s2}-casein (bovine casein 12.1%; caprine α_{s1}-I-casein 9.2%; caprine α_{s1}-II-casein 5.3%), α_{s1}-casein (type I): (bovine casein 0%; caprine α_{s1}-I-casein 4.0%; caprine α_{s1}-II-casein 0%); α_{s1}-casein (type II): (bovine casein 39.5%; caprine α_{s1}-I-casein 21.1%; caprine α_{s1}-II-casein 25.6%); β-casein: (bovine casein 37.2%; caprine α_{s1}-I-casein 51.6%; caprine α_{s1}-II-casein 60.6%); and κ-casein: (bovine casein 11.2%; caprine α_{s1}-I-casein 13.8%; caprine α_{s1}-II-casein 9.6%) [20].

2.3. Emulsion preparation

A corn oil-in-water emulsion was prepared as follows: an organic phase was prepared by diluting 2.5% (wt/wt) of the commercial lutein in corn oil. Soybean lecithin 0.5% (wt/wt) was dissolved in the corn oil. An aqueous phase was prepared by dispersing 1.0% (wt/wt) of lyophilized bovine casein or caprine casein into the aqueous buffer solution (5 mM phosphate, pH 7.0). A coarse emulsion of oil-in-water was prepared by mixing the organic phase (10%, wt/wt) and the aqueous phase (90%, wt/wt) using a hand-held homogenizer (Biospec Products, Inc., Bartlesville, OK, USA) at low speed. The coarse emulsion was then homogenized five times at 82.74 MPa (12,000 psi) through a high-pressure TC5 homogenizer (Stansted Fluid Power, Harlow, UK). The fine emulsion produced was then diluted (1:1, v/v) with buffer solution containing an antimicrobial agent [5 mM phosphate buffer at pH 7.0 and 1 mM (wt/v) thimerosal]. The final diluted emulsions that were used for the stability studies contained 5% (wt/wt) oil phase and 250 mg per liter of lutein. Resulting emulsions were stored in the dark at 5 and 15°C for 7 days.

2.4. Physical characterization of the emulsion

The particle size of the oil droplets in the lutein-enriched emulsions was measured at day 0 and day 7 after homogenization with a SALD-2101 laser diffraction particle analyzer (Shimadzu Corporation, Columbia, MD, USA). The charge of the oil droplets in the lutein-enriched

emulsion (zeta potential, ζ, mV) was measured at day 0 with a Zetasizer nano ZS (Malvern Instruments, Worcestershire, UK). Samples were diluted 100 times in 5 mM phosphate buffer at pH 7.0. All measurements were carried out in triplicate at 21 ± 1°C.

2.5. Chemical stability of lutein in the emulsion

The chemical stability of lutein was assessed by measuring the concentration of lutein in the lutein-enriched emulsions during storage at 5 and 15°C and analyzed right after production (0 day) and at 1, 2, 4, 6, and 7 days. The concentration of lutein was determined from absorbance measurements at 460 nm using a Beckman UV/visible model DU-530 spectrophotometer (Beckman Instruments Inc., Fullerton, CA, USA). To prepare the samples for the spectrophotometric measurements, the lutein-enriched emulsions were diluted 100 times in DMSO (50 μl of emulsion was diluted into 4.95 ml of DMSO). The DMSO was used because it dissolves lutein, oil, lecithin, and protein to form transparent solutions suitable for UV/visible analysis. The emulsion without lutein was used as a blank. A calibration curve was constructed by dissolving lutein standards in DMSO within a range from 0.5 to 5 mg/ml.

2.6. Statistical analysis

In each experiment, the results of triplicate analyses were used to test experimental variables. The data were analyzed by ANOVA using PRO GLM procedure of SAS (version 8.2, SAS Institute, Cary, NC, USA). The least significant test was used to determine significant differences among means at $p < 0.05$.

3. Results and discussion

3.1. Relationship between casein composition and emulsion interfacial properties

Caprine caseins have markedly higher content of β-casein than bovine casein. The specific oxidative stability found in emulsified lipids has been explained by the formation of a highly protective interface produced from β-casein as an emulsifier in the emulsions [23, 24]. Likewise, emulsions exert good protective effects on the carotenoids. Thus, it has been reported that the multilayer emulsions around the oil droplets can potentially reduce the amount of light reaching the carotenoid [19, 20]. A lutein dispersion was achieved using bovine casein or caprine caseins (caprine α_{s1}-I-casein and caprine α_{s1}-II-casein) as emulsifier in an emulsion beverage [19, 20]. The caprine casein emulsifier, in particular caprine α_{s1}-II-casein, in combination with arabinogalactan, a water-soluble polysaccharide, is noteworthy because this lutein dispersion system remains stable after light exposure during storage [20].

3.2. Physical stability of lutein emulsions prepared with bovine casein/lecithin and caprine casein/lecithin as emulsifiers

In order to understand the effects of bovine casein or caprine casein in combination with phospholipids i.e., soybean lecithin on the chemical degradation of lutein, three sets of emulsions with corn oil were made. The three sets were stabilized by bovine casein/lecithin,

caprine α_{s1}-I-casein/lecithin, or caprine α_{s1}-II-casein/lecithin. The mean particle diameters of oil droplets in the lutein-enriched emulsions stabilized by bovine casein/lecithin or caprine casein/lecithin ranged from 205.6 ± 2.3 to 208.9 ± 2.5 nm at pH 7.0 (**Table 1**). We observed no significant differences ($p > 0.05$) in the particle sizes of oil droplets in lutein-enriched emulsions with either bovine casein or caprine caseins during 0 and 7 days of storage at 21 ± 1°C.

The zeta potentials of the casein/lecithin-coated oil droplets in the lutein-enriched emulsions were negative at pH 7.0 (**Table 2**). The zeta potentials of the oil droplets in the lutein-enriched emulsions prepared with the two caprine casein/lecithin emulsifiers were not different ($p > 0.05$), showing mean values of −36.8 ± 1.8 mV for caprine α_{s1}-I-casein/lecithin and −35.9 ± 1.9 mV for caprine α_{s1}-II-casein/lecithin, respectively. The lower zeta potentials of the oil droplets in the lutein-enriched emulsions stabilized by the two caprine casein/lecithin emulsifiers compared to the lutein-enriched emulsions stabilized by bovine casein/lecithin emulsifier can imply a decreased 'net' negative charge [25]. Differences in the amino acids of side chains of κ-, α_s- and β-casein fractions in caprine whole caseins, where the less charged β-casein quantitatively dominates, could lead to less repulsive charge-charge interactions and this phenomenon can possibly explains why caprine caseins have lower zeta potentials (**Table 2**). On the basis of these findings, we can conclude that the lutein-enriched emulsions prepared with bovine casein/lecithin emulsifier and the two caprine casein/lecithin emulsifiers exhibited relatively good physical stability [13].

3.3. Chemical stability of lutein emulsions prepared with bovine casein /lecithin and caprine casein/lecithin as emulsifiers

Lutein degradation in the emulsions that were prepared with different emulsifiers was in the following order: bovine casein/lecithin > caprine α_{s1}-I-casein/lecithin > caprine α_{s1}-II-casein/

Particle size (nm)[1]			
Storage (d)	Bovine casein/lecithin	Caprine (α_{s1}-I)-casein/lecithin	Caprine (α_{s1}-II)-casein/lecithin
0	207.8 ± 2.0	208.9 ± 2.5	208.0 ± 2.1
7	205.6 ± 2.3	206.1 ± 2.4	205.7 ± 2.2

[1]Mean value ± Standard error.

Table 1. Particle size of oil droplets in lutein-enriched emulsions stabilized by bovine casein/lecithin or caprine casein/lecithin in phosphate buffer at pH 7.0 and 21 ± 1°C.

Emulsifier	**Zeta potential (mV)[1]**
Bovine casein/lecithin	−39.7 ± 1.8[a]
Caprine (α_{s1}-I)-casein/lecithin	−36.8 ± 1.8[b]
Caprine (α_{s1}-II)-casein/lecithin	−35.9 ± 1.9[b]

Means with different lowercase superscripts are significantly different ($p < 0.05$).

[1]Mean value ± Standard error.

Table 2. Zeta potential of oil droplets in lutein-enriched emulsions stabilized by bovine casein/lecithin or caprine casein/lecithin in phosphate buffer at pH 7 and 21 ± 1°C.

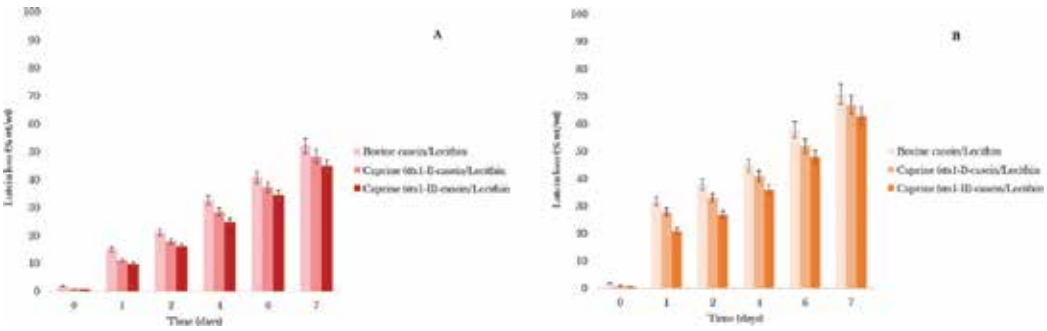

Figure 1. Lutein degradation in corn oil-in-water emulsions (pH 7.0) stabilized by bovine casein/lecithin, caprine (α_{s1}-I)-casein/lecithin or caprine (α_{s1}-II)-casein/lecithin at 5°C (A) and 15°C (B) as a function of storage time. Values represent the mean of three trials.

lecithin (**Figure 1**). The bovine casein/lecithin means on the bars are significantly different than the two caprine casein/lecithin means ($p < 0.05$) until four days of storage at 5°C (**Figure 1A**); the same tendency in lutein loss was observed during storage at 15°C (**Figure 1B**). Comparing the results of the two caprine casein/lecithin emulsifiers, the caprine α_{s1}-II/lecithin emulsifier overall showed lower means than caprine α_{s1}-I/lecithin emulsifier in both storage temperatures. In general, lutein degradation was faster in emulsions prepared with bovine casein/lecithin emulsifier than with the other two emulsifiers within 7 days of storage at 5 and 15°C (**Figure 1A** and **B**, respectively). The fact that lutein degradation was faster in the emulsions prepared with bovine casein/lecithin emulsifier compared to the emulsions prepared with caprine α_{s1}-I-casein /lecithin emulsifier or caprine α_{s1}-II-casein/lecithin emulsifier suggests that the interfacial layer formed by bovine casein/lecithin emulsifier was less efficient in protecting emulsified lutein against chemical degradation during storage at 5 and 15°C. The combination of caprine α_{s1}-I-casein or caprine α_{s1}-II-casein with soybean lecithin as emulsifiers resulted in a more favorable thickness of the interfacial layer thereby, slowing down the lutein degradation in these emulsions. This confirms that caprine caseins, which are 'rich' in their content of β-casein, formed a denser interfacial layer surrounding oil droplets and the possible role that the thickness of the interfacial layer played in the degradation of emulsified lutein. Furthermore, soybean lecithin, which is comprised of charged phospholipids such as phosphatidylinositol and phosphatidic acid [26], is more soluble in water and therefore, more easily absorbed at the oil-in-water interface thereby, producing a thicker interfacial layer protecting lutein.

4. Conclusions

The stabilizing role of phospholipids in emulsions is important in the absorption of lutein by the host. From the nutritional point of view consumption of a lutein-enriched beverage emulsion stabilized by bovine casein/lecithin emulsifier or caprine casein/lecithin emulsifier will be a valuable means to counterbalance the deficiency in consumption of fruits and vegetables; this approach will reduce the negative effects of lower ingestion of health-promoting carotenoids. Overall, these results indicate that a high chemical stability of lutein in corn oil-in-water emulsions can be achieved by altering the physical properties of the emulsion droplet

interface by the addition of emulsifiers such as caprine α_{s1}-I-casein/lecithin and caprine α_{s1}-II-casein/lecithin. The different effects of the bovine casein/lecithin and the caprine casein/lecithin emulsifiers on the stability of lutein is expected to be derived from the difference in the composition of bovine and caprine caseins, in particular the β-casein and its role at the interface of emulsion for the protection of emulsified carotenoids, specifically the lutein of the xanthophyll group. The charged phospholipids, phosphatidyl inositol and phosphatidic acid, in soybean lecithin may also decrease the degradation of lutein in oil-in-water emulsions by producing a thicker interfacial layer.

Acknowledgements

This work was supported by Evans-Allen funding to the Cooperative Agricultural Research Center through USDA Cooperative State Research Service.

Conflict of interest

The authors declare that they have no conflict of interest.

Author details

Adela Mora-Gutierrez*, Rahmat Attaie and Maryuri Núñez de González

*Address all correspondence to: admora@pvamu.edu

Prairie View A&M University, Cooperative Agricultural Research Center,
Prairie View, Texas, USA

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Chapter 10

Lutein and the Aging Eye

Shen Nian and Amy C.Y. Lo

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.79604

Abstract

Lutein is a carotenoid highly concentrated in the macula of the retina. Lutein cannot be synthesized and must be supplied in the diet, for example, dark green leafy vegetable and egg yolk. Lutein is believed to absorb blue light, leading to the protection of retina from light-related damage. It can also protect the retina against oxidative stress and inflammation. In fact, dietary and supplementary lutein have been shown to be associated with possible reduced risk of age-related macular degeneration, a leading cause of elderly blindness, attributed largely to lutein's antioxidant properties. Lutein is also beneficial as a nutritional supplement in preventing diabetic retinopathy. Moreover, lutein is very safe and widely used. In this chapter, we will discuss the basic chemistry of lutein; its uptake, transport, distribution, and functions in the normal eye. Lastly, the effects of lutein in age-related eye diseases will be summarized.

Keywords: macula, macular degeneration, vision, retinopathy, blindness

1. Introduction

Decades of research have indicated that abundant intake of carotenoid-rich food is correlated with the reduced risk of several age-related ocular diseases, for example, age-related macular degeneration (AMD) and diabetic retinopathy (DR). To date, among more than 1000 carotenoids discovered in nature, about 50 have been identified in the human diet [1]. However, only 25 dietary carotenoids and 9 of their metabolites have been found in human plasma, of which lutein, its stereoisomers zeaxanthin and meso-zeaxanthin are highly concentrated in the human retina [2].

Lutein is one of xanthophyll carotenoids (oxygen-containing carotenoids) which exist in the dark green leafy vegetables, yellow fruits and vegetables, and egg yolk [1]. Since animals are

not able to produce lutein, they need to depend on the dietary consumption. After absorption of lutein with fat, it is attached to the lipoprotein and then transported into the circulation; subsequently, with the serum concentration of 0.2 μm, lutein reached throughout the body and accumulated in the eye, especially in the retina, to serve certain biological functions [3]. In the human eye, the distribution of lutein varies. Lutein is found in higher quantities within the peripheral retina, retina pigment epithelium (RPE), choroid and ciliary body while exhibiting low concentrations in the iris and lens [2].

According to the most updated data from WHO, 253 million people suffer from vision impairment, and 81% people who are blind or have moderate or severe vision impairment are aged 50 or above [4]. A large number of studies have indicated that lutein plays an important role in decreasing the risk of AMD, the leading cause of blindness in the elderly people in the developed countries [5, 6]. Clinical trials have demonstrated that lower concentration of lutein in retina and serum was observed in DR patients when compared with patients without diabetes [7]. Moreover, DR patients receiving lutein and zeaxanthin supplements have shown improvement in visual acuity and contrast sensitivity, indicating a possible benefit in delaying the onset and development of DR [7]. In this chapter, we will introduce the background information of lutein, summarize its functions in the normal eye, and discuss the effects of lutein in age-related eye diseases.

2. Lutein

2.1. Chemistry and structure of lutein

Carotenoids are classified into two subgroups: carotenes, which are hydrophobic, consist of strictly hydrocarbons and xanthophylls, which are more hydrophilic, contain at least one oxygen atom in the polyene chain. The common characteristic of the carotenoid family is a $C_{40}H_{56}$ structure containing a long conjugated double-bound chain carrying the liner and cyclic alternatives. Lutein and zeaxanthin belong to the xanthophylls subgroup. They are characterized by the two hydroxyl groups attached to the end ionone rings in the nine conjugated carbon bounds polyene chain (**Figure 1**). The difference between lutein and its stereoisomer zeaxanthin is the position of the double bound in the terminal ring. In the human body, lutein and zeaxanthin could be transformed to each other via meso-zeaxanthin. Due to the presence of hydroxyl groups, lutein and zeaxanthin are more hydrophilic and polar in the serum and tissues, allowing them to react with oxygen produced in the liquid phase and scavenge reactive oxygen species (ROS) more efficiently. Due to the presence of chiral centers, lutein can exhibit eight stereoisomeric forms, of which (R,R,R) is mainly found in nature. On the other hand, zeaxanthin has three stereoisomeric forms, including (R,R), (S,S), and (R,S-meso).

2.2. Sources and safety of lutein

Lutein cannot be synthesized in human and lower animals, thus it must depend on the dietary supply in nature. Lutein, along with its structure isomer zeaxanthin is present in various natural foods, including kale, spinach, brussels sprout, broccoli, corn, lettuce, green peas,

http://dx.doi.org/10.5772/intechopen.79604

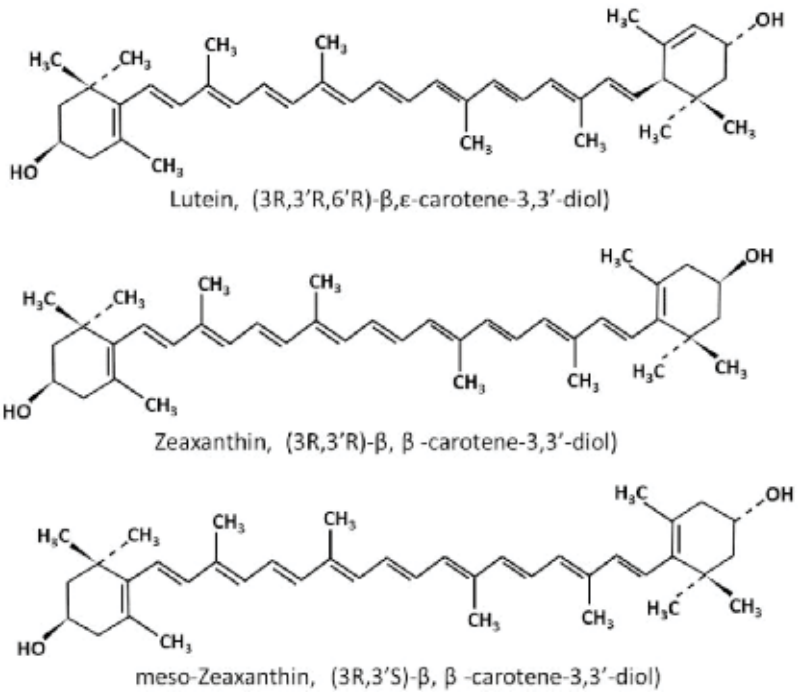

Figure 1. Chemical structures of macular pigments.

orange pepper, kiwi fruit, orange, zucchini, and squash. Dark-green leafy vegetables are the major source of lutein, especially in kale and spinach, containing 15,800–39,550 μg/100 g and 7040–11,940 μg/100 g, respectively [1]. There are 44 and 26 mg of lutein per cup of cooked kale and spinach, respectively [8]. However, the dietary origin of lutein varies in different countries, depending on the preference for specific foods. In Canada, lutein mostly comes from spinach, broccoli, lettuce, corn, and oranges; while in Germany, spinach and green leafy salads contribute almost 50% of the total lutein supply [1]. Egg yolks, although does not contain lutein as high as kale and spinach, are treated as a great source of xanthophylls due to the high fat content in eggs, resulting in increased bioavailability. The concentrations of lutein and zeaxanthin are 292 ± 117 μg/yolk and 213 ± 85 μg/yolk (average weight of yolk is 17–19 g), respectively [9]. It has been demonstrated that consumption of 6 eggs/week increased the macular pigment optical density (MPOD) significantly, while the serum concentration of total cholesterol, triacylglycerols, high density lipoprotein cholesterol, and low density lipoprotein cholesterol stayed unaffected [10]. Because of the limitation in separating and quantifying lutein and zeaxanthin, most researches and databases frequently report the combined data of these two compounds in food. Thus, it may result in the inappropriate estimation of lutein content in several xanthophyll-rich foods (e.g. oranges and grapes). The microalgae, especially the genus Chlorella, are also an important natural source of lutein. Compared to the marigold flower, the conventional source of lutein in market, microalgae have faster growth rate and can be obtained throughout the year. Therefore, they can be used as a potential source for commercial lutein products.

According to the National Health and Nutrition Examination Survey, the intake of lutein and zeaxanthin combined is approximately 1–2 mg/day in USA [11]. In addition, German adults consume 1.9 mg/day in average and 1.4 mg/day of lutein consumption was reported for Canadians [1]. No adverse effects were reported after the supplementation of dietary lutein up to 20 mg/day for 48 weeks, 30 mg/day for 120 days, and 40 mg/day for more than 8 weeks [12–14]. Animal studies have demonstrated similar results. For rat, uptake of lutein up to 35 mg/day for 8 weeks or 208 mg/kg/day for 13 weeks, or 639 mg/kg/day was not associated with any exposure-related toxicity and adverse events [15, 16]. Thus, lutein is recognized as Generally Recognized as Safe (GRAS) by FDA. Although there is no relationship between side effects and long term, high dose supplementation of lutein, the total intake should not exceed 20 mg/day according to the report from Council for Responsible Nutrition (CRN) in 2006 [17]. Generally, the recommendation dose of lutein supplements is 10 mg/day. A recent case report has demonstrated bilateral "foveal sparkles" in an Asian woman who has taken a 20 mg/day lutein supplements together with a high consumption of dietary lutein. After 7 months of discontinuous uptake of lutein supplements but insistence of her high-lutein diet, the crystal dissolved in the right eye, but still existed in the left eye [18]. However, it is worth noting that upon the population-based surveys, consumption of lutein has gradually declined in the USA and Europe. Therefore, actions should be taken to emphasize the importance of adequate intake of carotenoid-rich food, especially from dark-green leafy vegetables.

2.3. Absorption, metabolism, and transport of lutein

Since lutein and zeaxanthin are soluble in the fat, the absorption of these compounds follows a similar path like other lipophilic nutrients. After uptake of carotenoid-rich foods, xanthophylls are released from the food matrix with the aid of a variety of enzymes (e.g. esterase) and disperse in the stomach. The free xanthophylls then form micelles by incorporating with biliary phospholipids, bile salts, or dietary fats, which makes them more easily absorbed into the mucosal cells in the small intestine. Subsequently, they are transported from intestinal tract to the liver in the form of chylomicrons, where xanthophylls such as lutein and zeaxanthin are repackaged, carried by the relevant lipoproteins and released into the systemic circulation. In the circulation system, lipoproteins are responsible for transporting hydrophobic lipid including fat, plasma lipid, carotenoids, retinoids, etc. There are four types of lipoproteins: ultra-low density lipoproteins (ULDL), also known as chylomicrons; very low density lipoproteins (VLDL); low density lipoproteins (LDL); and high density lipoproteins (HDL). Compared to the non-polar carotenes such as lycopene and β-carotene, which are loaded onto VLDL and LDL, lutein and zeaxanthin are primarily transported by HDL. Both lutein and zeaxanthin are distributed in a variety of human tissues, but the distribution of them is not balanced among different tissues and organs. Retina, especially the macula, is regarded as the region where lutein, zeaxanthin, and its metabolite meso-zeaxanthin are concentrated, accounting for 25% of total carotenoids. Therefore, lutein, zeaxanthin, and meso-zeaxanthin are known as macular pigments (MPs), which play an important role in maintaining the normal functions of the eye. Although lutein is richest in the retina, it is also absorbed and distributed in other tissues such as adipose tissue in human body. It has been estimated that level of lutein in the retina was affected in obesity group, suggesting adipose tissue may compete with retina in terms of xanthophylls uptake [19].

There are several factors that affect the bioavailability of lutein and zeaxanthin, including Species of carotenoids, Linkage at molecular level, Amount of carotenoids ingested per meal, food Matrix, Effectors of carotenoid absorption and conversion, Nutrient status of the individual, Genetic factors, Host-related factors, and Interactions among these factors (short for SLAMENGHI) [20, 21]. Compared with β-carotene, the bioavailability of lutein supplied in a diet containing a large range of vegetables is much higher. The reason should be the presence of the hydroxyl groups in lutein, which makes it more polar and hydrophilic, leading to higher release of lutein into the aqueous medium. In addition, uptake of dietary fat together with lutein facilitated the formation of micelles and absorption of lutein in the gastrointestinal tract. It has been demonstrated that 3–5 g fat per meal is suitable to enhance the serum concentration of lutein [22]. However, lower bioavailability of lutein was observed when certain dietary fibers were present in foods. Sucrose polyester, a nonabsorbable fat substitute, impairs the ingestion of carotenoids such as lutein due to its preference for incorporation with nonabsorbable sucrose polyester rather than with micelles. The methods of food processing like heating, which improves release of lutein from food matrix, also influence the bioavailability of lutein. Furthermore, interactions between different types of carotenoids also affect the bioavailability. Studies have shown that lutein hampered the absorption of β-carotene, while β-carotene reduced the bioavailability of lutein [20].

3. Lutein and the eye

3.1. Lutein in the retina

The eye is made up of three separate layers, including the cornea and the sclera forming the outer fibrous layer; the uveal tract, which consists of the iris, ciliary body and choroid, forming the middle vascular layer; and the retina forming the inner neural tunic (**Figure 2**). In the central and posterior part of retina, there is an oval-shaped yellow area (approximately 5–6 mm in diameter) known as macula, which contains the highest concentration of photoreceptors. It is characterized by the yellow pigments that are entirely composed of lutein and zeaxanthin. The fovea, in the center of macula, is a small pit which is in charge of central vision and high-resolution visual acuity as a result of closely assembled cone cells. In addition, the retina consists of 10 layers from the outermost to the innermost, including RPE, photoreceptor cell layer, external limiting membrane (ELM), outer nuclear layer (ONL), outer plexiform layer (OPL), inner nuclear layer (INL), inner plexiform layer (IPL), ganglion cell layer (GCL), nerve fiber layer (NFL), and internal limiting membrane (ILM).

Although MPs exhibit high concentration in the retina, the distribution varies in different regions of the retina. The highest concentration of MPs is observed in the fovea at about 0.1–1 mM, which is over 100-fold higher than the rest area of retina. Moreover, the ratio of lutein and zeaxanthin also differs in different parts of retina. In the peripheral retina, lutein is the major carotenoids and the ratio of lutein to zeaxanthin is 2:1, whereas the ratio is reversed to 1:2 in the fovea.

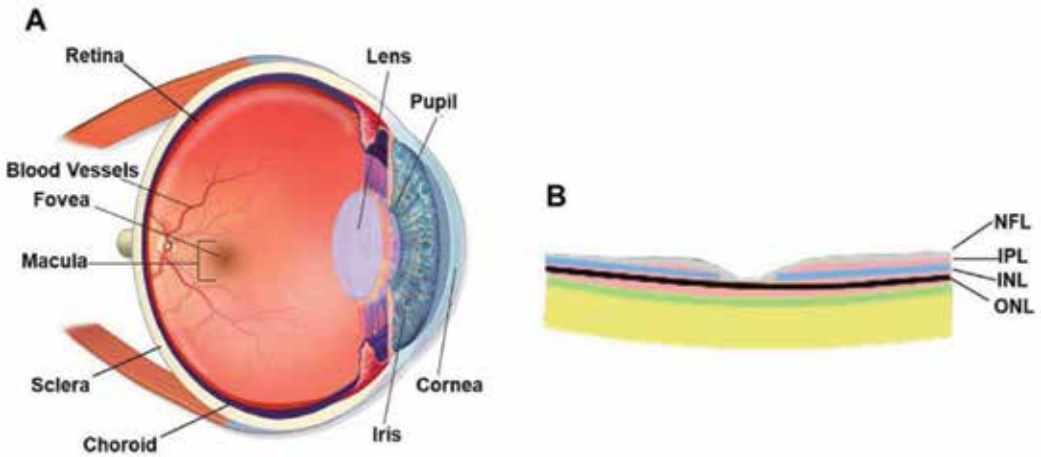

Figure 2. The human eye. (A) A schematic diagram demonstrating the anatomy of the human eye [23]. (B) A schematic image of optical coherence tomography (OCT) showing the vertical section of the center of the retina.

Of the 25 dietary carotenoids found in human tissues and blood, the selectively high rate of absorption and accumulation of lutein and zeaxanthin in human retina remained unclear until the discovery of specific macular carotenoid-binding proteins. Bernstein et al. have demonstrated that tubulin, a hydrosoluble protein, could bind to both lutein and zeaxanthin, and may be involved in the high distribution of MPs in the retina, but presented relatively low binding affinity and specificity. As a result, the research team continued to identify carotenoid-binding proteins with higher affinity and specificity. Subsequently, glutathione S-transferase P1 (GSTP1) was identified to bind zeaxanthin in the macula specifically compared to GSTM1 and GSTA1, the members of GST protein family [24]. GSTP1 was further confirmed to prevent lipid membrane from oxidation. In 2011, steroidogenic acute regulatory domain protein 3 (StARD3), one of lipid transfer-related protein family, was discovered as the lutein-binding protein [25]. Further studies need to be carried out to reveal more functions of StARD3. Generally, GSTP1 and StARD3 selectively bind zeaxanthin and lutein, respectively, leading to the high concentration and stabilization in human retina. In addition, the retinoid transporters including inter-photoreceptor retinoid-binding protein (IRBP) and retinol binding protein 4 (RBP4) are believed to be involved in the transport of MPs from circulation to retina [26].

3.2. Lutein and visual functions

3.2.1. Blue light filter

The peak value of MPs absorption is about 460 nm, which lies in the range of wave length of blue light (450–495 nm). Therefore, MPs can absorb 40–90% of incident high-energy, visible blue light depending on the concentration. The absorption offers protection from light-induced damages and reduction of light scatter in the retina. Junqhans A et al. [27] have investigated the efficacy of various carotenoids as the blue light filter using unilamellar liposomes with a

fluorescent dye which was excitable by blue light. Different carotenoids were incorporated with the lipophilic membrane, and fluorescence intensity was lower in carotenoid-containing liposomes than in control group when exposed to blue light, indicating a role of carotenoids as the blue light filter [27]. It is noted that lutein is more efficient in filtering blue light than zeaxanthin and meso-zeaxanthin because of the orientation in the biological membranes [27, 28].

3.2.2. Antioxidant function

A free radical is defined as a molecule, atom, or ion containing an unpaired electron. Because of the unpaired electron in the outer shell, the free radical is chemically highly reactive and unstable. Therefore, the free radical will react with other substances, even with themselves to reach steady state. Free radicals generated from oxygen are called reactive oxygen species (ROS), including superoxide anion (O_2^-), perhydroxyl radical (also known as hydroperoxyl radical, HO_2), and hydroxyl radical (OH) [29]. Superoxide can be inverted into only hydrogen peroxide (H_2O_2) and H_2O_2 together with singlet oxygen (non-radical compound) by enzymatic and non-enzymatic reactions, respectively [30]. Singlet oxygen, perhydroxyl radical, and hydroxyl radical are oxidants causing oxidation of protein, DNA as well as lipid peroxidation in cell membrane lipid bilayer, resulting in damages to the integrity of biological membrane and subsequently cell necrosis [29]. In physiological condition, production and detoxification of ROS are balanced in the body. However, when the balance is disrupted, no matter the increase in ROS generation or reduction of endogenous antioxidants, damages in the body occur. Thus it has been defined as "oxidative stress".

The retina is constantly exposed to ROS due to its high consumption of oxygen, conversion of light photons into electrochemical signals, and a number of mitochondria in rods. Massive blood supplies to the choroid in the retina make it the highest oxygen uptake tissue in the human body. Continuous exposure to the light photons, especially the blue light, triggers photo-oxidative reactions and damages DNA in RPE cells. Mitochondria, which are believed to be the major site for the generation of ROS, are rich in the inner segments of rod cells. It has been estimated that about 5% activated oxygen electrons in mitochondria could leak out as they go through the complicated electron transport chain, forming superoxide radicals [31]. Furthermore, a high content of polyunsaturated fatty acids in the outer segments of rods makes it more prone to peroxidation. In general, retina exhibits high susceptibility to ROS, resulting in irreversible oxidative damages.

Depending on the unique structure of MPs, one of the major biological functions of MPs in the retina is the prevention from oxidative damages via either physical quenching of singlet oxygen or chemical scavenging of free radicals. In the process of quenching of non-radical compound, such as singlet oxygen, the energy of singlet oxygen is transferred to the molecules of MPs, leading to excited triplet state of MPs and ground state of oxygen. Subsequently, the MPs in the triplet state dissipate the energy and return into the ground state. Since it is a physical mechanism, the structure of MPs is not changed, thus can be reused in the quenching cycles. It has been estimated that among carotenoids, lutein can react with singlet oxygen more strongly [32]. In contrast to physical mechanism, scavenging of ROS is achieved through chemical reactions in two ways. First, ROS accepts the missing electrons from MPs in which

electrons are available in the polyene chain, thus cannot induce oxidation of lipid, protein and DNA in cells. Second, lutein and zeaxanthin insert themselves into the cell membrane to pair the single electron in ROS, making the lipid bilayers more rigid. Lutein was found to insert into the biological membranes in perpendicular and parallel orientation, while zeaxanthin follows the perpendicular orientation in the lipid membrane [28]. It is the transmembrane alignment of MPs that reduce the susceptibility of lipid bilayers to oxidative injury and maintain the integrity and rigidity of biological membranes [33].

ROS is directly or indirectly involved in the most pathological processes observed in the retina, including inflammation, neuron degeneration, angiogenesis, or cell apoptosis. In the process of inflammation, excessive generation of ROS has been found to simulate many pro-inflammatory pathways. Moreover, oxidative injury is also associated with certain downstream signaling pathways in inflammation. Our research team has evaluated the anti-inflammatory effects of lutein in mouse model of ischemia/reperfusion and demonstrated that several pro-inflammatory factors, including nuclear factor-kappa B (NF-κB), interleukin 1β(IL-1β), and cyclooxygenase-2 (Cox-2), from Müller cells were significantly decreased in lutein-treated group when compared with control group, suggesting protection effects of lutein in retinal ischemia/reperfusion damage was achieved by its anti-inflammatory property [34]. Similarly, supplementation of lutein and zeaxanthin decreased NF-κB activity, while increased levels of erythroid 2-related factor 2 (Nrf2) and heme oxygenase 1 (HO-1), which are the key factors to initiate phase II antioxidant protection to eliminate oxidative stress, in rats fed with high fat diet [35].

3.2.3. Other functions

In addition to the functions mentioned above, lutein also plays an important role in maintaining other visual performance. A huge number of studies have shown that lutein and/or zeaxanthin, or in combination with other antioxidants have improved visual acuity and contrast sensitivity in healthy, young adults, in subjects with AMD at early and/or advanced stage, and in people with diabetes. High levels of MPs have been reported to decrease the influence of bright lights via quick recover from bright lights and improvement of ability to see in glare conditions. Daily uptake of lutein (20 mg/day) for a year increased visual contrast and glare sensitivity in healthy Chinese drivers, thus benefiting driving or other vision-related tasks performed at night [17]. Furthermore, MPs are able to speed conversion of photic impulses into electrical impulses in retina as well as the transmission to the visual cortex in the brain by keeping neurons in healthy state [36].

Furthermore, lutein has been shown to have neuroprotective effects in the retina. We have reported that in mouse model of ischemia/reperfusion, lutein decreased the expression of nitrotyrosine, and nuclear poly(ADP-ribose) (PAR) in GCL and INL, which are the markers for oxidative stress; thus exhibited protection effects on cell loss and cell apoptosis in inner retinal neurons [37]. Similar results were observed in the cerebral ischemia/reperfusion injury [38]. We further used the in vitro model of oxidative stress and hypoxia to evaluate the neuroprotective function of lutein in retinal ganglion cells. Our data revealed that lutein could protect ganglion cells from either H_2O_2-induced oxidative stress or $CoCl_2$ (cobalt

chloride)-induced chemical hypoxia [39]. Moreover, in the treatment of $CoCl_2$ on Müller cells, lutein not only improved cell viability and enhanced cell survival but also inhibited the formation of autophagosome [40]. In the rat model of retinal detachment, lutein preserved cells in ONL and rhodopsin expression, indicating its neuroprotective and anti-apoptotic effects [41]. In general, lutein could protect retinal neurons from hypoxia-induced injury.

4. Lutein and AMD

4.1. AMD

AMD is the leading cause of visual impairment in people 65 years and above in developed countries. It is a slowly progressive disease that affects the central retina or macula. As estimated by the United Nations, approximately 20–25 million people are affected by AMD across the world, and the prevalence inevitably rises with the increasing of aged populations. By the end of 2020, it is expected that only in the USA, the number of AMD patients will reach to almost 3 million [42].

AMD is a complicated, multifactorial ocular disease, and the exact etiology still remains unclear. However, a number of risk factors are thought to be related to the pathogenesis of AMD. Of all those factors, age is the most obvious risk factor. Both the incidence and prevalence of AMD increase with age. Many investigators revealed that the family members of AMD patients were more prone to develop this disease, demonstrating the genetic factors in the genesis of AMD. Furthermore, the incidence in Caucasians is higher than that in other ethnic populations. There is no apparent sex preference in AMD patients, although some studies have indicated that women may be more susceptible [43]. In addition to the unmodified factors mentioned above, several other factors that can be modified are also involved in the pathogenesis of AMD. Smoking is considered as a frequent environmental risk factor, which is proved to double the AMD risk through increasing the oxidative stress in the macula [44]. Excessive exposure to the sunlight can lead to lipid peroxidation on cell membranes. Hypertension, overweight or obesity, poor nutrition status, and cardiovascular diseases are also correlated with AMD.

AMD is classified into a non-exudative or atrophic (dry) form, accounting for 90% of AMD, and an exudative (wet) form, accounting for only 10% of AMD. The atrophic form is characterized by the accumulation of drusen under the macula formed by photo-oxidation of lipids plus proteins, and progressive degeneration of RPE cells in the macula, affecting central vision to varying degrees. The exudative form is associated with choroidal neovascularization (CNV) in the submacular area and subsequent retinal hemorrhage, leading to severe central vision loss.

The most destructive type of AMD is the exudative or wet form because of the sudden loss of vision. Therapies for the wet form of AMD mainly focus on halting the progression of CNV, of which intravitreal injection of anti-vascular endothelial growth factor (anti-VEGF) drugs have been widely adopted by ophthalmologists as a standard treatment due to the up-regulation of

VEGF in the development of CNV. Although it has been proved that anti-VEGF compounds can restrict growth of abnormal blood vessels, therefore making vision stabilized or even improved, the cost of each injection is relatively high and monthly intravitreal injection may be required for some patients. In contrast, the current treatments for non-exudative AMD are very limited. Hitherto, no medicine has yet approved for dry AMD in the world. Hence, strategies to delay the onset of this severe visual loss have been focused on the decrease of modified risk factors. Among these modified risk factors, oxidative stress is recognized as one of the major contributing factors in AMD. Since lutein is a powerful antioxidant that is highly concentrated in the retina, the effects of lutein on AMD have been widely investigated.

4.2. Clinical trials

4.2.1. Observational studies (dietary intake and serum concentrations of lutein)

Initially, the relationship between dietary consumption of lutein plus zeaxanthin and AMD has attracted much attention from researchers. Although the results of these studies were not consistent, most of them have demonstrated that a high dietary intake of lutein and zeaxanthin is correlated with lower risk of AMD. A systematic review and meta-analysis was performed to analyze six longitudinal cohort studies and found that intake of lutein and zeaxanthin had different effects on early and late AMD [45]. Consumption of these dietary xanthophylls was strongly associated with the reduced risk of late AMD (relative risk [RR] 0.74; 95% confidence interval [CI] 0.57, 0.97) and neovascular AMD (RR 0.68; 95%CI 0.51, 0.92). However, an inverse relation was not observed between dietary intake of lutein plus zeaxanthin and the risk of early stage AMD. In the Age-Related Eye Disease Study (AREDS) report No. 22, 4519 subjects aged 60–80 years were included for the analysis of association between dietary lutein plus zeaxanthin and AMD status. Compared with the lowest quintiles of dietary lutein and zeaxanthin intake, there were a 55, 35, and 27% lower probability to develop geographic atrophy, neovascular AMD, and large or extensive intermediate drusen, respectively [6]. Similarly, in the Blue Mountains Eye Study, Tan and colleagues [46] evaluated dietary intake of different antioxidants in relation to the long-term risk of incident AMD in Australia, and indicated a 65% reduction in neovascular AMD between the individuals having highest and lowest uptake of lutein and zeaxanthin. The data from Rotterdam Study further revealed the influence of both genetic and environmental risk factors on AMD, demonstrating a protective role of high intake of dietary antioxidants including lutein and zeaxanthin, β carotene, omega-3 fatty acids, and zinc, in AMD at early stage [47].

As early as 1993, Eye Disease Case-Control Study (EDCCS) has reported the direct correlation between serum levels of lutein plus zeaxanthin and AMD risk, demonstrating a distinct risk reduction of neovascular AMD to one-third in subjects with highest serum concentration of lutein and zeaxanthin when compared to those in the lowest group [48]. The research performed by Delcourt et al. [49] has further confirmed that AMD was significantly related with plasma lutein and zeaxanthin and tended to be associated with plasma lutein. A recent study carried out in an Irish population-based sample was in accord with the results discussed above, presenting a lower plasma concentration of lutein in AMD patients no matter whether they were aware of their suffering from AMD or not [50].

4.2.2. Observational studies (MPs levels in the retina)

In addition to dietary intake and serum concentration of lutein and zeaxanthin, MPs level in the retina was also inversely associated with the risk of AMD. In a case-control study, the actual amounts of lutein and zeaxanthin in donor retinas with and without AMD were measured. Levels of lutein and zeaxanthin in three concentric areas (inner, medial, and outer) centered on the fovea were markedly lower in AMD donor retinas than these in control donor retinas, especially in the outer area, where logistic regression analysis suggested that donors in highest quartile of MPs levels had an 82% lower risk for AMD when compared with those in the lowest quartile after adjustment of age and sex [51]. This is the first report showing the decreased retinal levels of lutein and zeaxanthin in AMD patients, which was consistent with above findings concluded from diet and serum xanthophylls concentrations. Subsequently, MPOD, an indicator for MPs levels in retina in vivo, has been widely studied between healthy individuals and AMD patients. There was a MPOD decline in healthy eyes as the individuals aged, and MOPD in healthy eyes at high risk of AMD was significantly lower than those at no such risk [52, 53]. Moreover, Bernstein and his co-workers [54] evaluated MPs levels in relation to the incidence of AMD using noninvasive resonance Raman spectroscopy, and found 32% reduction of retinal lutein and zeaxanthin levels in AMD versus normal participants. However, it was notable that lower MPOD has also been linked with other risk factors for AMD, such as smoking and family history of this disease [55]. This result further supported the hypothesis that lutein and zeaxanthin could prevent or delay the development of AMD by increasing MPOD.

4.2.3. Interventional studies (supplementation of lutein)

Observational results in relation to AMD have triggered a mass of interests in assessing effects of lutein supplementation on the risk of AMD. The supplementation trial was first reported in the Lutein Antioxidant Supplementation Trial (LAST) study [56]. This was a prospective, double-masked, placebo-controlled, randomized study to evaluate supplementation of lutein alone or lutein with other antioxidants, vitamins, and minerals in 90 atrophic AMD patients. After 12 months, higher MPOD, improved visual acuity and contrast sensitivity were observed in both of these groups than in placebo group. However, longer duration of the study, larger number of samples, and both genders are needed to examine the long-term effects of lutein or the combination of lutein with other nutrients in the treatment of dry AMD. Three years later, LASTII was performed to further analyze the specific factors that affected MPOD, including age, baseline levels of MPs, and combination of lutein and other antioxidants [57]. There was an increase in MPOD with supplementation, while a moderate reduction of MPOD was observed without supplementation. Patients with lowest baseline MPOD value were most likely to have a dramatic increase in MPOD than those with medium to high baseline MPOD during one-year supplementation of lutein or lutein with other nutrients. The reason might be the saturation mechanism that had an impact on the retinal transportation and stabilization of MPs.

In the Combination of Lutein Effects in the Aging Retina (CLEAR) study, Murray et al. [58] supplemented the patients at early stage AMD with 10 mg lutein esters per day for up

to 1 year. MPOD increased significantly after 8 months of supplementation, and plasma concentration of lutein increased by 1.8-fold to 7.6-fold compared to the baseline values. In addition, visual acuity in lutein group remained stable while the declined visual acuity was exhibited in the placebo group, indicating that stabilization of visual acuity was probably maintained by the elevated MPs level. These results were in accord with the study carried out by Ma et al. [13, 59], who demonstrated the significant improved responses of multifocal electroretinogram (mfERG) in lutein group and in lutein plus zeaxanthin group, and tended to be related to the increase of MPOD.

In a randomized controlled clinical trial (known as the Carotenoids with Coantioxidants in Age-Related Macular Degeneration [CARMA] study), 433 patients who were identified to be at highest risk of progression to advanced AMD received a daily supplementation of lutein, zeaxanthin, vitamin C, vitamin E, zinc, and copper at the duration of 12–36 months [60]. Visual acuity was increased in the intervention groups at 12 months, but not statistically significant until 24 months. Contrast sensitivity was slightly improved without significance. Level of MPs declined steadily in the placebo group, while MPs was increased in the supplemented groups throughout the whole trial. A rise of plasma concentration of all contents in the supplementation, especially lutein and zeaxanthin, was observed after 6 months. Although the increase of all antioxidants in blood was not associated with VA improvement, higher serum level of lutein slowed the progression of AMD. Fewer eyes progressed to severe state in the intervention group than in the placebo group (15.3 vs. 18%).

The Age-Related Eye Disease Study 2 (AREDS2) was a randomized, placebo-controlled, double-masked trial conducted in the USA from 2006 to 2012 [61]. The participants involved in AREDS2 were subjects aged 50–85 years at risk for progression to advanced AMD with bilateral large drusen or large drusen in one eye and advanced AMD in the fellow eye. The main objective of AREDS2 was to evaluate the effects of lutein, zeaxanthin, and omega-3 long-chain polyunsaturated fatty acids adding into the AREDS formulation, which was composed of vitamin C (500 mg), vitamin E (400IU), β-carotene (15 mg), and zinc (80 mg zinc oxide) with copper (2 mg cupric oxide). After the follow-up of 6.5 years on average, the AREDS supplements was proved to significantly decrease the development to advanced AMD, and an approximately 25% reduction in risk of progressing to late AMD was observed at 5 years [62]. Moreover, the beneficial effects of this AREDS formula were found to persist for 5 more years of follow-up after the end of this trial [63]. However, supplementation of β-carotene may lead to the increased risk of lung cancer in cigarette smokers [64, 65]. In addition, 80 mg/day zinc is out of tolerance for individuals and high amount of zinc was associated with increased genitourinary complications [62, 66]. Therefore, AREDS2 supplementation was changed as follows: the primary randomization was composed of AREDS formulation with (1) lutein (10 mg) + zeaxanthin (2 mg), (2) fish oil (350 mg DHA + 650 mg EPA), (3) lutein + zeaxanthin + EPA + DHA, and (4) placebo; the secondary randomization included (1) AREDS formulation, (2) AREDS formulation with low zinc (25 mg), (3) AREDS formulation without β-carotene, and (4) AREDS formulation with low zinc (25 mg) and without β-carotene. Former and current smokers are randomly assigned to the groups without β-carotene. In the primary analysis, no further reduced risk of developing advanced AMD was observed when comparing each of the treatment groups with placebo group [61]. Although the preconceived goal of 25% incremental

improvement over the original effective AREDS formulation was not achieved, analyses of patients with lutein and zeaxanthin supplements versus those without lutein and zeaxanthin supplements demonstrated a 10% decrease in the risk of progression to advanced AMD in the group with lutein and zeaxanthin [67]. Furthermore, the analyses of comparing participants receiving lutein and zeaxanthin with those receiving β-carotene were performed. The risk of developing advanced and neovascular AMD was significantly decreased in the group with lutein and zeaxanthin. In analyses restricted to eyes with bilateral large drusen at baseline, protective effects of lutein and zeaxanthin were more prominent. Therefore, considering beneficial effects of lutein and zeaxanthin as well as harmful effects of β-carotene on smokers, replacement of β-carotene with lutein and zeaxanthin in AREDS2 formula is preferred.

4.3. Basic research

Several animal models that mimic the pathological changes in AMD have been adapted to further study effects of lutein and zeaxanthin on AMD in human. Apolipoprotein E-deficient mice (apoE-/-), a well-established genetic mouse model of hypercholesterolemia, exhibited deposits on the basal laminar, vacuoles in RPE cells, and increased Bruch's membrane thickness, which are similar to the retinal changes in human AMD. These alterations were associated with the elevation of retinal lipid peroxidation and VEGF expression [68]. Administration of lutein alone could partially prevent the retinal alterations, and decrease expression level of VEGF but with no statistical significance was observed in comparison with controls. However, the combination of lutein and multivitamin and glutathione complex ameliorated all the morphological changes observed in retina and decreased VEGF levels significantly [68, 69]. In the mouse models that show similar retinal changes in human dry AMD, AREDS2 formulation prevented accumulation of liposomes and lipofuscin in RPE, loss of photoreceptors, and increased ONL thickness. In molecular level, mRNA expression levels of pro-inflammatory factors including inducible nitric oxide synthase (iNos), tumor necrosis factor-α(TNF-α), Cox-2, IL-1β, and angiogenic factors such as VEGF was significantly lower in AREDS2-treated group than control groups [70]. Furthermore, supplementation of lutein and zeaxanthin from grapes or marigold extract attenuated a reduction of a-wave amplitude in ERG, suggesting protective effects on photoreceptor functions [71]. Mouse model for the wet form of AMD is induced by laser photocoagulation, characterized by the formation of CNV. It has been reported that pretreatment of lutein significantly inhibited macrophage infiltration in CNV and expression of pro-inflammatory molecules such as NF-κB that subsequently resulted in significant suppression of CNV development [72].

Data from in vitro studies were also consistent with findings from animal experiments. Addition of lutein and other antioxidants (zeaxanthin, lycopene, or α-tocopherol) led to a significant decrease in formation of lipofuscin in RPE cells from bovine and rabbit under hypoxia condition [73]. Oxidative damages in ARPE-19 cells (a human RPE-derived cell line) were induced by the challenge of H_2O_2, leading to decreased cell viability, increased cell apoptosis, and ROS generation. Pretreatment of lutein protected ARPE-19 cells from these oxidative injuries and accumulation of Alu RNA, which is related to the pathogenesis of AMD [74, 75]. In addition, G2/M phase arrest triggered by oxidative damage was reversed by lutein in a dose-dependent manner [75].

5. Lutein and DR

5.1. DR

DR is the most common microvascular complication in diabetes. For individuals with diabetes aged 40 years and older, the estimated number of DR patients is 93 million around the world, of which 17 million are proliferative DR and 28 million are vision-threatening DR [76]. In the USA, approximately 2.8 million individuals may develop sight-threatening DR. DR used to be considered as high of prevalence in western countries, however, there is a rising prevalence of DR occurred in Asian countries (such as China and India) due to the changes in economics, diet habit, physical exercise, and so on.

According to the presence of microvascular lesions in the retina, DR is classified into early nonproliferative stage, featured with microaneurysms, vascular tortuosity, retinal hemorrhages, "hard" lipid exudates and microinfarcts in the NFL (known as the "cotton wool spots"), and late proliferative stage, characterized by the formation of new aberrant fragile blood vessels in the retina. Another important manifestation of DR is diabetic macular edema present at any stage, causing the abnormal thickening of retina and cystoid edema in the macula. Diabetic macular edema, together with retinal neovascularization, is the major cause of vision loss in patients with diabetes.

DR is considered to be a multifactorial disease with its exact pathogenesis being still uncertain. It has been proved that increased blood glucose concentration is the key factor in the onset and development of DR, leading to exacerbation of hypertension and dyslipidemia, overproduction of ROS that subsequently damages the retina. Oxidative stress disrupts retinal mitochondrial functions by inner membrane oxidation and mitochondrial DNA damage, which in turn lead to apoptosis of retinal capillary cells [77]. In addition, inflammation is also involved in the pathogenesis of DR. Increased retinal pro-inflammatory mediators such as intracellular adhesion molecule-1 (ICAM-1), TNF-α, and IL-1β are induced in diabetes. In clinical studies, presence and progression of DR were associated with the increased plasma levels of TNF-α, IL-1β and VEGF [78]. VEGF is also an important factor in the development of DR, which leads to the increased permeability of retinal blood vessel and angiogenesis.

Current major treatments for DR include laser photocoagulation and/or intravitreal injection of anti-VEGF drugs. However, these therapies are expensive, invasive, and need to visit ophthalmologists at certain intervals. Therefore, lutein, a powerful antioxidant, may be adopted as a natural, noninvasive, long-term medication for DR.

5.2. Clinical trials

Although tremendous clinical studies have been performed to evaluate the relationship between carotenoids and diabetes, only a few have examined the effects of carotenoids including lutein and zeaxanthin on DR.

In a prospective study, Hu et al. [7] demonstrated that plasma levels of lutein and zeaxanthin was significantly lower in nonproliferative DR patients than in subjects without diabetes. Similarly, Brazionis et al. [79] assessed the serum concentration of different carotenoids in relation to the DR in type 2 diabetes and found significant lower blood levels of combined lutein and zeaxanthin and lycopene in diabetic patients with DR compared to these without DR. Moreover, a significant inverse correlation between odds of DR and plasma concentration of combined lutein and zeaxanthin and lycopene was shown in this study [79].

Retinal level of xanthophylls was examined via measurement of MPOD. Patients with DR had decreased MPOD in comparison with diabetic patients without DR [80]. Furthermore, MPOD in individuals with type 2 diabetes was significantly lower than in subjects with type 1 diabetes and normal control, despite similar dietary uptake of carotenoids among these groups [81]. The lower MPOD in patients with type 2 diabetes may probably be associated with obesity, where enhanced competition of lutein and zeaxanthin intake with higher body fat occurred (retina vs. adipose tissue). In type 2 diabetic patients with or without DR, MPOD was inversely associated with glycosylated hemoglobin, a more stable indicator for diabetes [82].

On basis of the above observational studies, influence of lutein and zeaxanthin supplementation on DR was evaluated. Administration of lutein (6 mg/day) plus zeaxanthin (0.5 mg/day) for 3 months led to a significant increase of serum lutein and zeaxanthin level, as well as the improvement in visual acuity, contrast sensitivity, and diabetic macular edema in nonproliferative DR [7]. This study was consistent with the results conclude from supplementation of 10 mg/day lutein for 36 weeks in patients with nonproliferative DR [83]. A recent study has shown an increased thickness in the central fovea and improved retinal response density after 2-year supplementation of combined lutein (10 mg/day), zeaxanthin (2 mg/day), and mesozeaxanthin (10 mg/day) in type 2 diabetic patients without DR, indicating beneficial effects of xanthophylls on visual functions in diabetes [84].

5.3. Basic research

In animal models of DR, lutein has been reported to have beneficial effects on affected retinal layers and visual functions by its antioxidant, anti-inflammation, and neuroprotection properties. The animal models used to study DR usually include mice or rats injected with alloxan or streptozotocin (STZ) that can directly destroy β cells in pancreas to halt insulin production and subsequently induce diabetes, and spontaneous diabetic mice (db/db mice), a type 2 diabetic animal model.

In alloxan-induced DR mice, oxidative makers (NF-κB and malondialdehyde) were increased, while antioxidants including glutathione (a powerful endogenous antioxidant) and glutathione peroxidase were decreased. Decreased b-wave amplitude in ERG was also observed. Supplementation of lutein (0.2 mg/kg) prevented all the diabetes-induced changes [85]. The same results were reported in STZ-induced diabetic rats after administration of lutein together with DHA. Moreover, prevention of histological changes including

decreased ONL, INL, and GCL thickness was also observed [86]. In STZ-induced murine models, Sasaki et al. [87] demonstrated changes in oxidative stress-related factors (increase of ROS, extracellular signal-regulated kinase activation, and depletion of brain-derived neurotrophic factor), retinal morphological changes (reduction of IPL, INL, and ganglion cells), and visual functions (decrease of oscillatory potentials in ERG, indicating dysfunction of neurons in inner retina). Likewise, lutein supplements (0.1 mg wt/wt) restored all the diabetes-induced damages in the retina [87]. Similarly, supplements containing lutein, zeaxanthin, omega-3 fatty acids, and other nutrients demonstrated protective effects on progression of DR in STZ-induced diabetic rats. Decreased ROS level, mitochondrial DNA damage, and inflammatory factors such as VEGF, IL-1β, and NF-κB, as well as reduction of retinal apoptosis, abnormal capillaries formation were demonstrated in treatment group compared with placebo control group. Furthermore, nutrient supplements ameliorated decreased amplitudes of a- and b-wave in ERG, suggesting prevention of retinal functions in diabetic rats with DR [88].

Wolfberry, a Chinese traditional fruit consumed for eye protection, is high in zeaxanthin (176 mg/100 g) and lutein (5 mg/100 g). In db/db mice, wolfberry elevated lutein and zeaxanthin levels in retina and liver, attenuated mitochondrial dysfunction and endoplasmic reticulum stress caused by hyperglycemia-induced oxidative stress, and restored retinal structure abnormalities [89, 90]. Furthermore, Lutein and zeaxanthin was able to protect cultured ARPE-19 cells from a high glucose challenge through the similar mechanisms, suggesting wolfberry's protection effects were at least partly due to high contents of lutein and zeaxanthin [89].

6. Conclusions

Lutein, synthesized in plants but not in mammals, is absorbed and highly accumulated in the macula. The uneven distribution of lutein is thought to afford a distinct function in the retina. Up to now, numerous epidemiological studies have demonstrated that higher levels of lutein in diet and plasma are correlated with lower risk of AMD, especially the late stage of AMD. Randomized and controlled clinical trials such as AREDS2 have reported that supplementation of lutein alone or with other nutrients leads to the increase of MPOD, improvement of visual functions, and decreased risk of progression to advanced AMD, especially the wet AMD. Laboratory experimental data also indicate that lutein can protect impaired retina by filtering blue light, attenuating oxidative stress and inflammation, and enhancing neuroprotection. However, the optimal dose of lutein, the best ratio of lutein and other antioxidants, therapeutic effects at different stages of AMD, adverse effects with even longer intake of lutein supplements in high dose, and the relationship between MPOD and AMD at different phases need to be further investigated in future studies. Although there are several studies assessing the effects of lutein on DR in clinical trials and laboratory experiments, further evaluations to fully understand its protective role in DR are necessary.

Acknowledgements

This work is supported by the Project of Dominant Discipline Construction in Universities of Shaanxi Province-Basic Medicine, Shaanxi Province, China awarded to Xi'an Medical University and Health and Medical Research Fund, Hong Kong (03142256) awarded to Amy C.Y. Lo.

Conflict of interest

The authors declare no conflict of interest.

Author details

Shen Nian[1] and Amy C.Y. Lo[2]*

*Address all correspondence to: amylo@hku.hk

1 Department of Pathology, Xi'an Medical University, Xi'an, Shaanxi Province, China

2 Department of Ophthalmology, LKS Faculty of Medicine, The University of Hong Kong, Hong Kong

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Chapter 11

Monitoring Crop Carotenoids Concentration by Remote Sensing

Wenjiang Huang, Xianfeng Zhou,
Weiping Kong and Huichun Ye

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.78239

Abstract

Assessment of carotenoids (Car) content provides a valuable insight into clarifying the mechanisms of plant photoprotection and light-adaption and is critical for stress diagnoses in plants. Due to their small proportion in the overall total pigment content and to the overlapping of spectral absorption features with chlorophylls (Chl) in the blue region of the spectrum, accurate estimation of Car content in plants, from remotely sensed data, is challenging. Previous studies made progress in Car content estimation at both the leaf and canopy level with remote sensing techniques. However, established spectral indices and methods for Car estimation in most studies that generally rely on specific and limited measured data might lack predictive accuracy for Car estimation and lack sensitivity to low or high Car content in various species and at different growth stages. In this chapter, a new carotenoid index (CARI) was proposed for foliar Car assessment with abundant simulated leaf data and various measured leaf reflectances. Detailed analysis on the mechanism, formation and performance of the new spectral index on Car retrieval was presented. Analysis results suggested that accurate nondestructive estimation of foliar Car content with CARI could be achieved at the leaf scale, through remote sensing techniques.

Keywords: carotenoids, retrieval, hyperspectral, remote sensing, radiative transfer model

1. Introduction

Photosynthetic pigments, mainly including chlorophylls (Chl) and carotenoids (Car), are of tremendous significance in the biosphere. Their photosynthetic function could provide necessities, such as oxygen and organic matters, for plant and mammal survival [1]. Generally,

chlorophylls, composed of chlorophyll a (Chl a) and chlorophyll b (Chl b), represent the principal class of pigments responsible for light absorption in photosynthesis [2]. Carotenoids, that include carotenes and xanthophylls, are the second major group of plant pigments [1]. They are part of the essential structures of the photosynthetic antenna and reaction center and help stabilize chlorophyll-protein complexes [3, 4]. Besides their function in photosynthesis, previous studies suggest that the assessment of the variation of Car and of their ratio to Chl could shed light on the understanding of photoprotection, photosynthetic acclimation and photosynthetic efficiency in plants [5–10]. Within the plant growth cycle, a normal decrease in Chl indicates that plants are affected by environmental stresses, while the variation of Car reflects the physiological status of vegetation [10]. For instance, it has been observed that Car content would change when plants are in sun-intense and high-temperature conditions, or when nitrogen availability is low or at the onset of leaf senescence [5]. Therefore, quantitative estimation of Car content is extremely useful in order to clarify the mechanisms of photoprotection and light-adaption and for early diagnosis of stress in plants.

The absorption features of Car in the visible range make it possible for Car content retrieval with remote sensing techniques. Based on its absorption features, researches on Car content estimation at both the leaf and canopy level with spectroscopic analysis have been conducted in recent years [11]. With ratio analysis of reflectance spectra (RARS) method, Chappelle et al. [12] suggested that reflectance at the 500 nm wavelength correlated best with Car content, and the reflectance was less affected by other pigments. Thus, they proposed a ratio analysis of reflectance spectra ((RARSc, R_{760}/R_{500}) for Car assessment. Research conducted by Datt [13] indicated that the maximum sensitivity of reflectance to variation in pigment content was in the green band region at 550 nm and in the red-edge region at 708 nm; a reflectance band ratio index (RBRI) ($R_{672}/(R_{550} \times R_{708})$) was then proposed for pigment content estimation, which had a good correlation with Car content. Based on the reflectance of the Car absorption band at 470 nm, Blackburn [14] put forward two spectral indices with the optimal wavebands 470 and 800 nm, that is, pigment specific simple ratio (PSSRc) and pigment specific normalized difference (PSNDc), for Car estimation at the leaf level. Gitelson et al. [11] found that the first-order derivative reflectance around 510 nm was the most sensitive to Car content. They established two spectral indices, that is, carotenoid reflectance index 550 (CRI_{550}) and carotenoid reflectance index 700 (CRI_{700}), for foliar Car content assessment. Based on a conceptual three-band model, Gitelson et al. [15] further put forward green carotenoid index (CAR_{green}) and red-edge carotenoid index ($CAR_{red\text{-}edge}$) with three bands located at 510–520 nm, 690–710 nm (560–570 nm for CAR_{green}) and a NIR band, for Car retrieval at the leaf level. Research conducted by Hernández-Clemente et al. [16] indicated that vegetation canopy structure severely affected the performance of CRI_{550} for Car content assessment at the canopy level. A simple ratio index (SRcar, R_{515}/R_{570}) was then proposed and it showed good correlation with Car content at both leaf and canopy levels.

Overall, previous studies have indeed made much progress in Car content estimation both at the leaf and canopy level; nevertheless, most of the research focused on establishing spectral indices or models for Car content retrieval, with limited measured datasets. These limited data might not be generic enough in order to provide a robust method of assessing

http://dx.doi.org/10.5772/intechopen.78239

Car composition and distribution, at a range of phenological stages and leaf structures. Spectral indices or models based on these datasets might be site- or species-specific, their robustness and capability deserve further investigation when applied to a wide variety of plant leaves and conditions. Thus, to develop robust spectral indices or models for Car content retrieval with spectroscopic techniques, the quality of the training dataset, the selection of the optimal wavelengths and the availability of an independent dataset for the validation are critical [17].

Radiative transfer models (RTMs) are effective tools to clarify the mechanism describing the relationships between spectral reflectance and plant parameters. They provide an analysis of the remote sensing signal based on a robust understanding of the physical, chemical and biological processes, allowing to assemble rapidly abundant simulation datasets [18]. In recent years, the RTMs have been used extensively for various applications on the vegetation studies [19]. Based on simulated data at the leaf and canopy level with leaf model PROPSECT [20] and multilayer canopy model Scattering by Arbitrary Inclined Leaves (SAIL) [21], there are researches on leaf biochemical parameters retrieval, such as leaf chlorophylls content (LChl), leaf mass per area (LMA) and leaf carotenoids content (LCar), with spectral indices methods and RTMs inversion [18, 22–24]. However, less attention was given to the application of RTMs in Car content retrieval than to Chl content assessment. For foliar Car content estimation, leaf model PROSPECT could simulate abundant leaf level data through combining plant biochemical parameters, which could be used for the investigation of optical characteristic of Car and other pigments and for quantitative evaluation of estimating results of foliar Car content with different spectral indices as well. In addition, for assessment of leaf Car content with plant canopy spectra, canopy spectra were influenced by more than biochemical parameters, canopy structure, illumination and observation geometry, and soil background properties affected canopy spectrum as well [25]. Among these factors, leaf area index (LAI), one of the key parameters describing the canopy structure, and the soil background, has a large effect on canopy reflectance signals [26, 27]. Utilization of PROSAIL model (coupled by leaf model PROSPECT and canopy model SAIL) could generate an extensive canopy level dataset useful for better understanding the relationship between canopy geometry, background environment and canopy reflectance, thus it could shed light on the effect of LAI and soil background on foliar Car content assessment and provide basis for an accurate and robust LCar estimation with spectral index methods.

Therefore, the goal of this chapter is to propose a nondestructive method to assess LCar with remote sensing techniques, through developing an accurate and robust LCar estimation index, using simulated and measured datasets based on their absorption features in the visible spectrum. The specific objectives were to: (1) establish a new carotenoid index (CARI) for LCar estimation, assess and compare its performance with published carotenoid indices using leaf level simulated data obtained from PROSPECT-5; (2) evaluate the capability and robustness of the new CARI and published carotenoid indices with various leaf level measured data including the widely used ANGERS dataset and field survey data; (3) clarify the effect of LAI and soil background on LCar assessment with the CARI using an extensive synthetic dataset obtained from PROSAIL and measured data at the canopy scale.

2. Materials and methods

2.1. Field experiment

A field experiment was designed and conducted in 2004 to collect measured data at both the leaf and canopy level for foliar Car content assessment. The experiment site was located at the National Experimental Station for Precision Agriculture (40°10.6′ N, 116°26.3′ E), Beijing, China. Winter wheat (*Triticum aestivum* L.) was used in this experiment, and 21 cultivars of winter wheat were grown in plots of 30 × 5.4 m size in the experiment site. Fertilization and irrigation were applied according to local standard practice so as to provide nonlimiting conditions. During the whole growing season, field measurements were conducted on specific growth stages including booting (April 28), head emergence (May 11), pollination (May 28) and milk development (June 08). For each growth period, different cultivars were used for sampling at both the canopy and leaf levels.

2.2. Field measurements

2.2.1. Reflectance spectrum measurements

On each sampling date, a 1 × 1 m area of winter wheat was first selected for canopy reflectance measurements, with an Analytical Spectral Devices (ASD) FieldSpec spectrometer (Analytical Spectral Devices, Inc., Boulder, CO, USA) under clear, blue-sky conditions between 10:00 and 14:00 h (Beijing Local Time). Measurements were obtained from a nadir position at approximately 1.3 m above the wheat canopy and taken by averaging 10 scans. Reflectance spectra were derived relative to a 0.4 × 0.4 m white reference panel, which was placed horizontally just above the wheat canopy.

Crop aboveground biomass from the 1 m^2 area was collected immediately after canopy spectral measurements, kept in a portable refrigerator and then transferred to a laboratory for leaf reflectance measurement and biochemical analysis. Leaf spectra were obtained using the ASD spectrometer coupled with a Li-Cor 1800-12 integration sphere (Li-Cor, Inc., Lincoln, NE, USA). For each leaf sample, measurements were made on five different areas (avoiding leaf veins). The sample was illuminated by a focused beam, which was produced by a Li-Cor 787 halogen lamp light source (6 V, 10 W, 3100 K), and the radiation that was captured by the spectrometer was the average reflected radiation within the Li-Cor 1800-12 integration sphere [28].

2.2.2. Plant measurements

Laboratory analyses were made on the 1 m^2 quadrat wheat samples just after leaf spectral measurement. LCar, leaf dry mass and LAI were measured according to standard procedures. Leaf dry mass was determined drying the samples in an oven at 70°C for 48 h, leaf Car content was determined using an L6 ultraviolet-visible spectrophotometer (INESA, China). Chlorophyll a (Chl a), chlorophyll b (Chl b) and total carotenoids' (Car) concentrations were calculated with Eqs. (1) to (3) [29]; the unit of total carotenoids could then be converted into content unit, that is, mass per unit leaf dry weight (mg/g), and concentration unit, that is, mass per unit leaf area (μg/cm^2), using data on the volume of leaf pigment extract, the leaf dry weight and the leaf disc area, with Eqs. (4) and (5):

$$\text{Chl}a\ (\text{mg/L}) = 12.21 \times A_{663} - 2.81 \times A_{646} \quad (1)$$

$$\text{Chl}b\ (\text{mg/L}) = 20.13 \times A_{646} - 5.03 \times A_{663} \quad (2)$$

$$Car\ (\text{mg/L}) = (1000 \times A_{470} - 3.27 \times Chla - 104 \times Chlb)/229 \quad (3)$$

$$Car\ (\text{mg/g}) = [Car(mg/L) \times V_T(ml)] / [\text{DW}(g) \times 1000] \quad (4)$$

$$Car\ (\mu\text{g/cm}^2) = [Car(mg/g) \times DW(g)] / \text{leaf area}(cm^2) \quad (5)$$

where A_X is the absorbance of the extract solution at wavelength x, V_T (ml) is the volume of leaf pigment extract solution and DW (g) is the leaf dry weight.

LAI was determined using a dry weight method [30]. Leaf segments of approximate area 0.06 m^2 were cut from the central part of about 30 leaves selected from all the green leaves in the 1 m^2 quadrat as standard leaves for LAI calculation. Both the standard leaves and the remaining leaves were oven dried at 70°C to constant weight and weighed. LAI was calculated as Eq. (6):

$$\text{LAI} = (S_r \times W_t)/(S_l \times W_r) \quad (6)$$

where S_r (m^2) is the area of the standard leaves, W_t (g) is the total dry weight of the 1 m^2 quadrat sampled leaves, S_l is the sampled land area (m^2) and W_r (g) is the dry weight of the standard leaves.

2.3. ANGERS dataset

Besides the winter wheat measured data, the ANGERS dataset, which contains various plant species and different growth conditions, was also used. The dataset was collected in 2003 on temperate plants at the National Institute for Agricultural Research (INRA), ANGERS, France. It contains leaf directional-hemispherical reflectance and transmittance spectra measured at 1 nm resolution from 400 to 2400 nm using ASD FieldSpec instruments equipped with integrating spheres. Chlorophyll *a* and *b* (Chl), total carotenoids (Car), water (also named equivalent water thickness (EWT)) and dry matter (also named leaf mass per area (LMA)) content are available for each sample [18].

2.4. Simulated datasets

PROSPECT-5 simulates leaf directional-hemispherical reflectance and transmittance from 400 to 2500 nm with six input variables: LChl, LCar, LMA, EWT, leaf structure parameter (N) and brown pigments (Cbrown). Generally, pigments absorb light in the visible range (400–760 nm), whereas water has a high absorbance in the near-infrared band (1000–2500 nm). Dry matter and refractive index variations extend through the whole wave range (400–2500 nm) [18]. Since the goal was to estimate leaf Car content mainly from visible wavebands, and the

Models	Parameters	Values
PROSPECT-5	Leaf chlorophyll content (LChl, μg/cm²)	10/20/30/40/50/60/70/80/90/100
	Leaf carotenoid content (LCar, μg/cm²)	2/4/6/8/10/12/14/16/18/20
	Leaf structure parameter (N)	1.6/1.7/1.8/1.9/2.0
	Leaf mass per area (LMA, g/cm²)	0.002/0.003/0.004/0.005/0.006
	Equivalent water thickness (EWT, cm)	0.012
	Brown pigments (Cbrown)	0
4SAIL	Leaf area index (LAI)	1/2/3/4/5/6/7/8
	Leaf angle distribution (LAD)	Spherical
	Soil moisture parameter (P_{soil})	0/0.5/1
	Solar zenith angle (SZA, °)	30
	View zenith angle (VZA, °)	0
	View azimuth angle (VAA, °)	0
	Fraction of diffuse incident radiation	0.23
	Hot spot effect	0.15

Table 1. Input parameters for PROSPECT-5 and 4SAIL models used for leaf and canopy reflectance modeling.

visible range was unaffected by EWT, the EWT value was kept fixed at the average EWT value of ANGERS dataset. The range of variation of LChl, LCar, N and LMA obtained from ANGERS dataset was used in simulations. Detailed values for the input parameters used in PROSPECT-5 simulations are shown in **Table 1**.

With PROSPECT-5 model, 2500 leaf reflectance simulations could be obtained by random combination of the parameters values. To avoid unrealistic combinations, we made use of the content ratio of Car to Chl to restrain the combinations. Statistics of the content ratio of Car to Chl in the ANGERS data show that the ratio values ranging from 0.1 to 0.6 account for 97% of the samples. This criterion was then used to eliminate invalid combinations and finally 1700 leaf reflectance were kept. To investigate the effect of LAI and soil background on LCar assessment, LAI values were set to change from 1 to 8 with a step of 1; soil moisture parameter values were set to vary from 0 to 1 with a step of 0.5. Other input variables were fixed and defined based on [26]. Input values used for 4SAIL are shown in **Table 1**. Then, 40,800 canopy reflectance were obtained using the PROSAIL model.

2.5. Spectral indices

Published spectral indices for Car content assessment were summarized in **Table 2**. In addition to these existing spectral indices, a new spectral index for foliar Car content estimation was proposed based on the spectral absorption features of Car and Chl observed with the leaf level simulated dataset. The correlation between Car and Chl with reflectance ranging from 400 to 800 nm was first investigated. **Figure 1a** shows that the correlation peak region is located in the range

Spectral index	Equation	Reference
Ratio analysis of reflectance spectra (RARS*c*)	R_{760}/R_{500}	[12]
Pigment specific simple ratio (PSSR*c*)	R_{800}/R_{470}	[14]
Pigment specific normalized difference (PSND*c*)	$(R_{800} - R_{470})/(R_{800} + R_{470})$	[14]
Reflectance band ratio index (RBRI)	$R_{672}/(R_{550} \times R_{708})$	[13]
Plant senescence reflectance index (PSRI)	$(R_{678} - R_{500})/R_{750}$	[8]
Carotenoid reflectance index (CRI_{550})	$(R_{510})^{-1} - (R_{550})^{-1}$	[11]
Carotenoid reflectance index (CRI_{700})	$(R_{510})^{-1} - (R_{700})^{-1}$	[11]
Red-edge carotenoid index ($CAR_{red\ edge}$)	$[(R_{510})^{-1} - (R_{700})^{-1}] \times R_{770}$	[15]
Green carotenoid index (CAR_{green})	$[(R_{510})^{-1} - (R_{550})^{-1}] \times R_{770}$	[15]
Photochemical reflectance index (PRI)	$(R_{570} - R_{531})/(R_{570} + R_{531})$	[31]
Modified photochemical reflectance index (PRI_{m1})	$(R_{512} - R_{531})/(R_{512} + R_{531})$	[32]
Simple ratio (SR_{car})	R_{515}/R_{570}	[16]
Carotenoid index (CARI)	$R_{720}/R_{521} - 1$	[33]

R_λ is the reflectance value at wavelength λ.

Table 2. Spectral indices selected for LCar assessment.

500–540 nm for Car, and band 521 nm showed the maximum correlation, suggesting that reflectance in this range is very sensitive to Car content [11]. Besides, the range of its maximum sensitivity overlapped with Chl absorption features (**Figure 1a**). For Chl, the correlation extended from 400 to 760 nm and two strong correlation peaks were observed in green and red-edge regions.

To establish a new spectral index for LCar estimation, Band 521 nm was chosen on the consideration that it had the highest correlation with LCar (R^2 = 0.607, RMSE = 3.144 μg/cm^2, **Figure 1b**), although a strong correlation with LChl also existed. Band 720 nm was selected to reduce the influence of Chl on LCar estimation since it showed the highest relationship with LChl (R^2 = 0.906, RMSE = 7.981 μg/cm^2, **Figure 1c**). The proposed new carotenoid index (CARI, R_{720}/R_{521}–1) was then established, based on the formula of chlorophyll indices (i.e., $CI_{red\text{-}edge}$ and CI_{green}). Simulated and measured datasets were then used to investigate its capability and robustness for LCar assessment.

2.6. Statistics analysis

Linear regression models between leaf carotenoids content and spectral indices derived from simulated and measured datasets were obtained using the SPSS 18.0 software (SPSS Inc., Chicago, IL). A k-fold (k = 6) cross-validation procedure was used to evaluate the performance of spectral index methods using ANGERS and experimental data, and all the selected spectral indices were tested using the same k-fold partitions. The overall performances of these models were evaluated by statistics including a coefficient of determination (R^2), root mean square error (RMSE), relative RMSE (RRMSE) and mean absolute error (MAE).

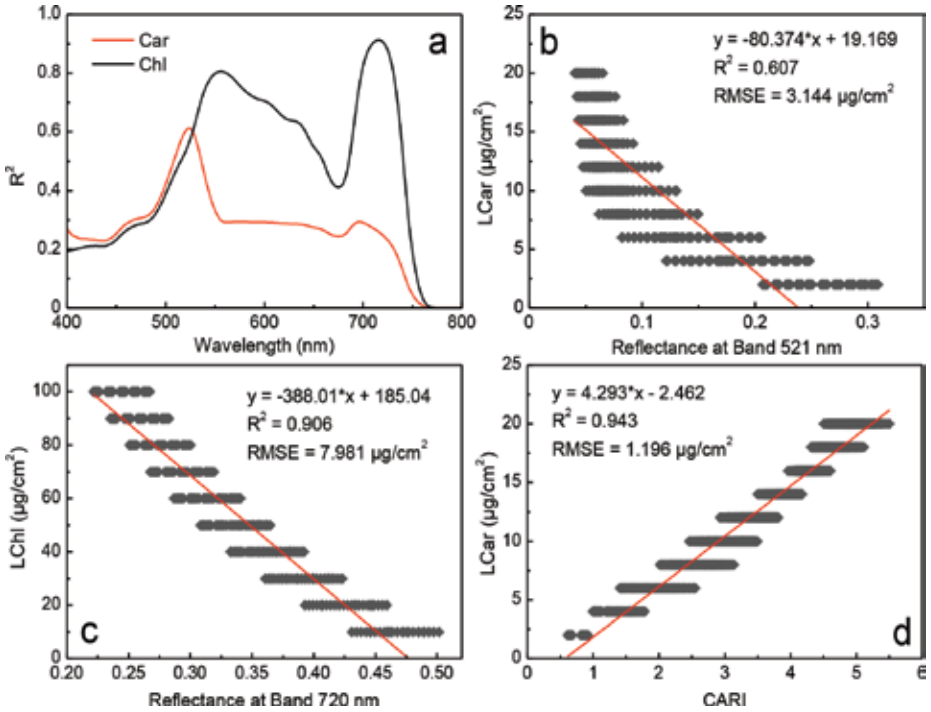

Figure 1. (a) R^2 curves for LCar (LChl) versus leaf reflectance within the wavelength range from 400 to 800 nm (b) Correlation between band 521 nm and LCar (c) Correlation between band 720 nm and LChl and (d) linear relationship between CARI and LCar.

3. Leaf car content assessment

3.1. Simulation results at the leaf level

Based on PROSPECT-5 leaf simulations, correlation between CARI and LCar is presented in **Figure 1d**. Results showed that CARI had a significant linear relationship with LCar ($R^2 = 0.943$, RMSE = 1.196 μg/cm²), indicating that CARI index was accurate in estimating LCar with leaf-simulated data. Nevertheless, relationships between established spectral indices and LCar varied in **Figure 2**. Among these established spectral indices, the carotenoid indices, that is, CRI_{550}, CRI_{700}, $CAR_{red\text{-}edge}$ and CAR_{green}, proposed by Gitelson et al. [11, 15] showed the highest correlation ($R^2 > 0.77$, the RMSE < 2.40 μg/cm²) with LCar. However, when LCar values were high, correlations between these indices and LCar presented with large dispersion, suggesting that these indices might be not sensitive to high LCar values (>15 μg/cm²). Compared with CRI_{550} and CRI_{700}, adding of a near infrared band (770 nm) in $CAR_{red\text{-}edge}$ and CAR_{green} did not improve the estimation accuracy of LCar. Correlation between RARSc and LCar was general ($R^2 = 0.603$, RMSE = 3.160 μg/cm²), and when LCar values were higher than 10 μg/cm², the correlation showed an obvious nonlinear trend. RBRI index was less correlated with LCar ($R^2 = 0.165$, RMSE = 4.584 μg/cm²), and the scatter plot of RBRI versus LCar showed

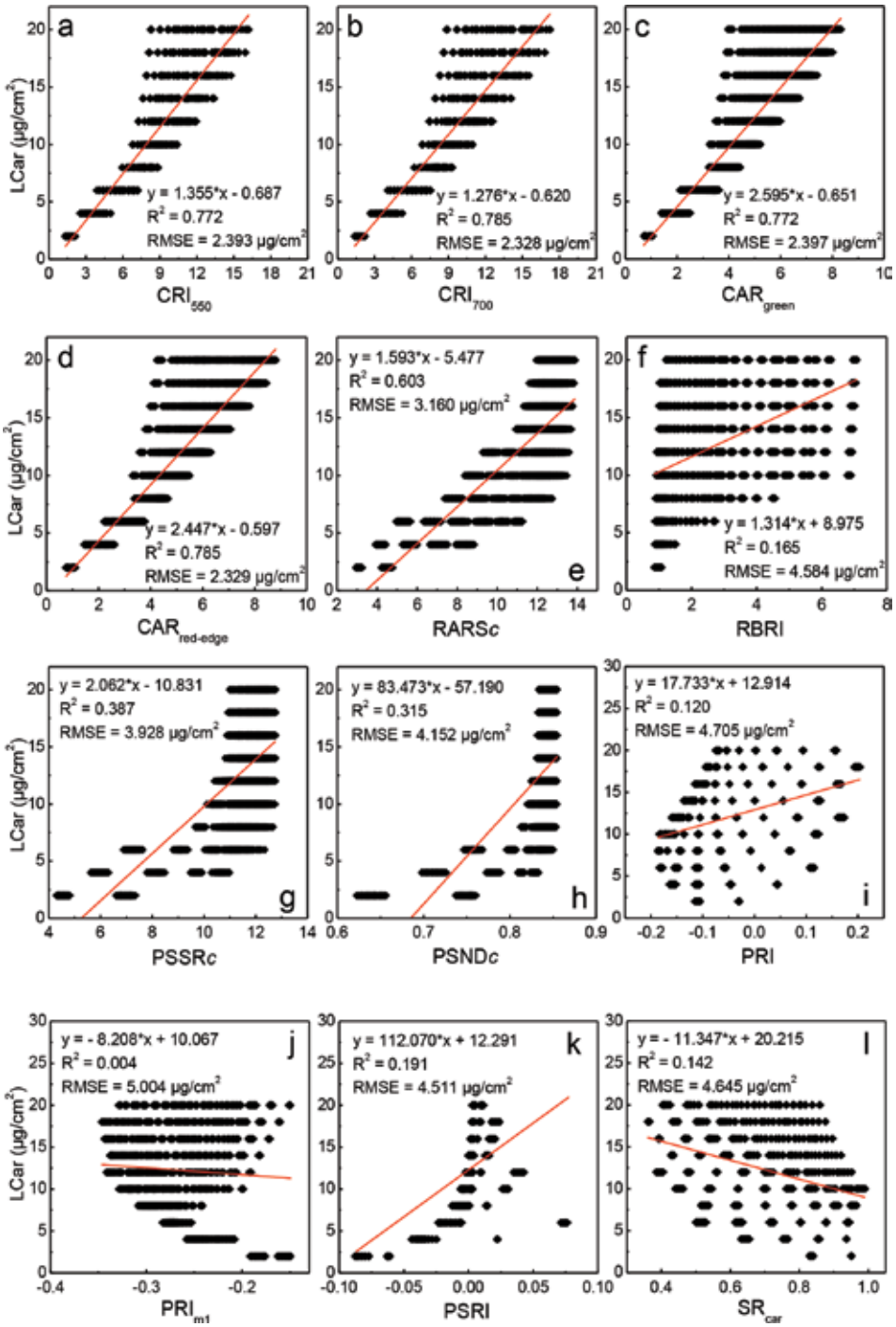

Figure 2. Relationships between published spectral indices and leaf carotenoids content from leaf level data simulated with PROSPECT-5.

large dispersity. PSSRc and PSNDc had low correlations with LCar. Compared with PSNDc, PSSRc showed a slightly better correlation with LCar (R^2 = 0.387, RMSE = 0.387 μg/cm^2). However, when LCar values exceeded 10 μg/cm^2, PSSRc and PSNDc present obvious nonlinear correlations with LCar (**Figure 2g** and **h**). Correlation between PRI and LCar was poor (R^2 = 0.120, RMSE = 4.705 μg/cm^2), and the scatter diagram showed obvious dispersion (**Figure 2i**). As for its modified version PRIm1, it showed almost no correlation with LCar, indicating that PRIm1 might not be suitable for the estimation of LCar. PSRI showed a low correlation with LCar (R^2 = 0.191, RMSE = 4.511 μg/cm^2), and the correlation was nonlinear. Different from these vegetation indices, SRcar showed a lower negative correlation with LCar (R^2 = 0.142, RMSE = 4.645 μg/cm^2), and the scatter diagram also showed strong dispersity.

3.2. Car assessment using ANGERS dataset

First, the ANGERS dataset was used to analyze the capability of different spectral indices in estimating LCar. Performance of different spectral indices in LCar assessment is shown in **Table 3**. The estimation accuracy of $CAR_{red\text{-}edge}$ and CAR_{green} in LCar assessment was slightly better than that of CRI_{550} and CRI_{700}. However, compared with the simulated results, these indices showed rather poor performance in LCar retrieval with the ANGERS data. RARSc exhibited good performance in LCar retrieval with a R^2 value of 0.438 and a RMSE value of 3.792 μg/cm^2. Although RBRI showed poor correlation with LCar in the leaf-simulated data, its estimation accuracy in LCar retrieval was the highest in the ANGERS data (R^2 = 0.727, RMSE = 2.640 μg/cm^2). Compared with PSNDc estimation results, estimation accuracy of PSSRc is relatively high, which is consistent with the foliar simulated results. PRI showed low accuracy in LCar estimation (R^2 = 0.199, RMSE = 4.527 μg/cm^2), while PRIm1 also showed poor estimation results.

Index	Rank	R^2	RMSE (μg/cm^2)	MAE (μg/cm^2)	RRMSE (%)
CRI_{550}	10	0.139	4.693	3.363	54.179
CRI_{700}	11	0.138	4.696	3.413	54.217
CAR_{green}	8	0.184	4.568	3.199	52.732
$CAR_{red\text{-}edge}$	7	0.190	4.550	3.232	52.524
RARS*c*	**3**	**0.438**	**3.792**	**2.757**	**43.781**
RBRI	**1**	**0.727**	**2.640**	**1.808**	**30.475**
PSND*c*	9	0.167	4.617	3.472	53.303
PSSR*c*	**4**	**0.310**	**4.201**	**3.142**	**48.499**
PRI	6	0.199	4.527	3.295	52.267
PRI_{m1}	12	0.075	4.869	3.505	56.215
PSRI	13	0.002	5.057	3.796	58.377
SR_{car}	5	0.213	4.489	3.117	51.820
CARI	**2**	**0.545**	**3.413**	**2.345**	**39.400**

Table 3. Cross-validation results for LCar assessment using ANGERS data.

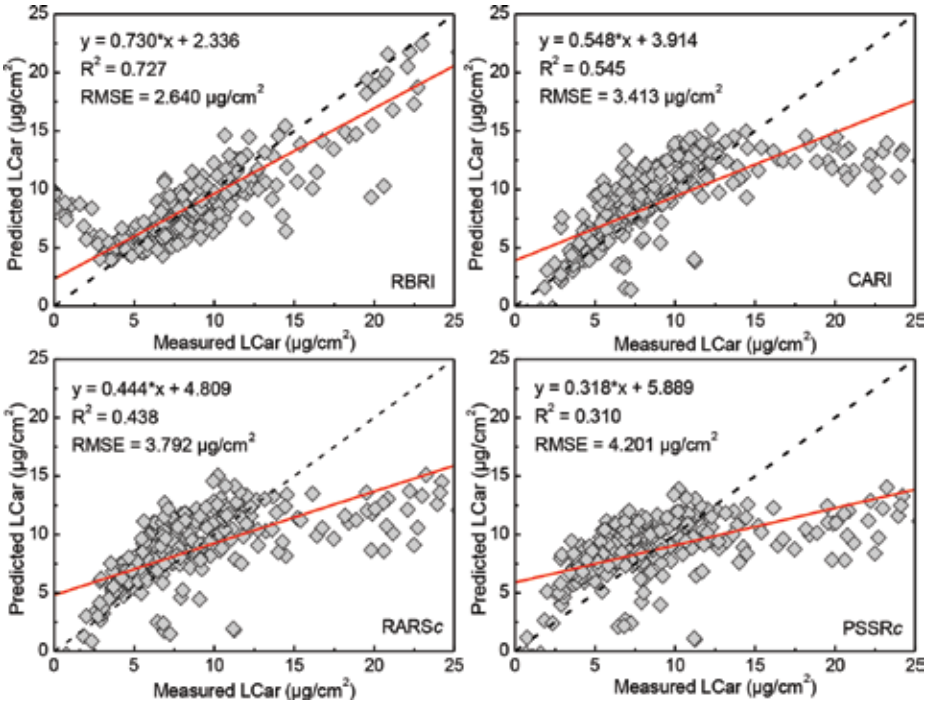

Figure 3. Scatterplots of measured LCar versus predicted LCar for spectral indices with ANGERS dataset. Dashed lines indicate 1:1 lines.

Index	Rank	R^2	RMSE (μg/cm²)	MAE (μg/cm²)	RRMSE (%)
CRI_{550}	12	0.124	2.395	1.998	28.531
CRI_{700}	13	0.046	2.533	2.121	30.171
CAR_{green}	7	0.411	1.941	1.637	23.122
$CAR_{red\ edge}$	9	0.344	2.050	1.739	24.417
RARS*c*	**2**	**0.674**	**1.443**	**1.130**	**17.192**
RBRI	10	0.222	2.234	1.777	26.614
PSND*c*	**4**	**0.618**	**1.563**	**1.239**	**18.623**
PSSR*c*	6	0.579	1.641	1.299	19.544
PRI	**1**	**0.710**	**1.369**	**1.092**	**16.305**
PRI_{m1}	11	0.125	2.373	1.814	28.268
PSRI	8	0.388	2.063	1.539	24.570
SR_{car}	5	0.614	1.571	1.144	18.713
CARI	**3**	**0.639**	**1.520**	**1.166**	**18.106**

Table 4. Cross-validation results for LCar estimation with wheat leaf level field data.

Among all the indices, PSRI had the lowest estimation accuracy, possibly due to its insensitive to LCar. The estimation accuracy of SRcar generally (R^2 = 0.213, RMSE = 4.489 μg/cm^2) ranks fifth in all estimation results. Compared with these existing spectral indices, the estimation accuracy of CARI was accurate (R^2 = 0.545, RMSE = 0.545 μg/cm^2), second to RBRI, showing that CARI data can be used to accurately estimate LCar in the ANGERS data.

Based on the estimation results of these spectral indices in LCar retrieval with the ANGERS dataset, the scatter diagrams of the best four ranking spectral indices were presented in **Figure 3**. The results showed that compared with other indices, the fitting line of the scatterplot of RBRI is closer to the 1:1 straight line (the slope of the fitting line is 0.730).In addition, RBRI index was more sensitive to higher leaf carotenoid content (>15 μg/cm^2). The CARI index also showed good estimation results, except for the samples that had LCar values greater than 15 μg/cm^2, and the estimated values of most sample points were evenly distributed around the 1:1 straight line with the measured values. Compared with RBRI, CARI was more sensitive to lower LCar values (<3 μg/cm^2), but it showed a slight "saturation effect" on high LCar values (>15 μg/cm^2). RARS and PSSRc indices also showed satisfactory estimation results. Similar to CARI, these indices were not sensitive to higher LCar values (>15 μg/cm^2).

3.3. Car retrieval with leaf level experimental data

Leaf level experimental data of winter wheat were used to further investigate the capability of the above spectral indices in LCar estimation. Results in **Table 4** showed that the estimation accuracy of CRI_{550} and CRI_{700} is the worst. $CAR_{red\text{-}edge}$ and CAR_{green} have slightly improved LCar estimating accuracy. RARSc presented an excellent estimation result (R^2 = 0.674, RMSE = 1.443 μg/cm^2), which is consistent with its estimation results with the ANGERS data. Different from its good estimation accuracy in ANGERS data, RBRI showed poor estimation accuracy in the experimental data (R^2 = 0.222, RMSE = 2.234 μg/cm^2). PSNDc and PSSRc have a performance with good results in LCar estimation with the experimental data (R^2 > 0.57, RMSE < 1.65 μg/cm^2). Compared with its poor estimation results in the ANGERS data, PRI showed the highest estimation accuracy of LCar in the experimental data (R^2 = 0.710, RMSE = 1.369 μg/cm^2). PRIm1 showed poor estimation results in the experimental data, which was consistent with the ANGERS data. Compared with the ANGERS data, the estimation accuracy of PSRI and SRcar in LCar with the experimental data had slightly improved. Similar to its performance with the ANGERS data, CARI had high estimation accuracy in LCar retrieval with the experimental data (R^2 = 0.639, RMSE = 0.639 μg/cm^2), showing that CARI was accurate and robust for LCar estimation with different leaf level datasets.

Similar to the results with the ANGERS dataset, scatter diagrams of the best four ranking spectral indices were presented in **Figure 4**. The results showed that the estimation results obtained by PRI were the best. LCar values estimated by PRI and the measured values were concentrated near the 1:1 line, and the slope of the scatter plot was 0.76. In addition, RARSc, CARI and PSNDc also showed good estimation results. Unlike the ANGERS data (**Figure 3**), The LCar values of the leaf level experimental data were in the range from 3.05 to 12.59 μg/cm^2, and LCar was in low to moderate numerical range. According to **Figure 4**, the majority of LCar values of the samples were around 10 μg/cm^2, this was mainly because most of the samples that were collected at the booting, head emergence and pollination stages had little LCar variation.

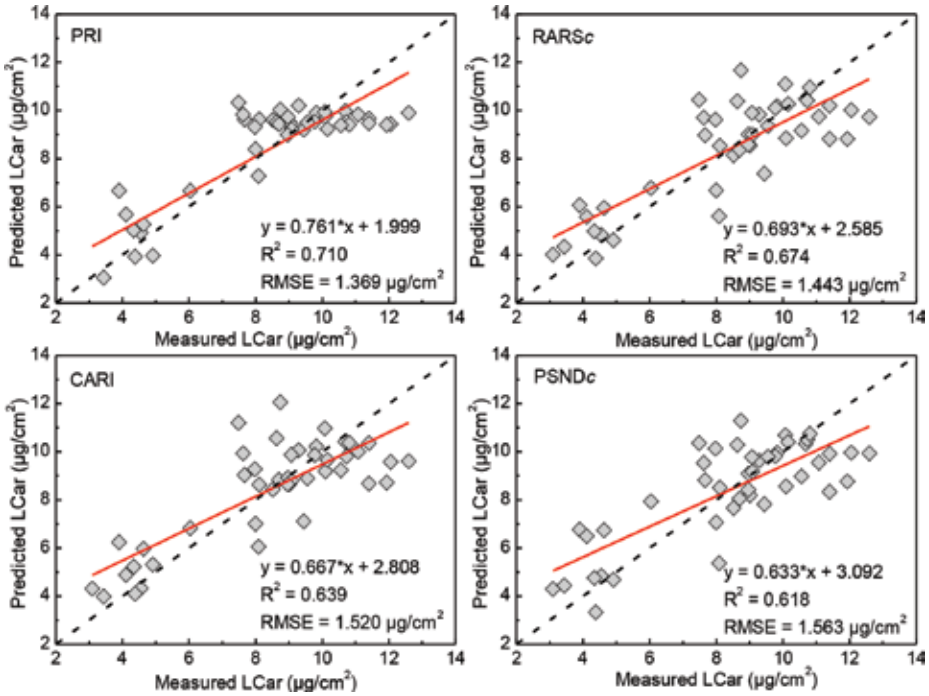

Figure 4. Scatterplots of measured LCar versus predicted LCar for spectral indices with leaf level experimental data. Dashed lines indicate 1:1 lines.

3.4. Assessing CARI for LCar retrieval with canopy spectra

The above results with leaf level measured data showed that CARI was accurate and robust in LCar estimation. Canopy level simulations and measured data were then used to further explore the effect of LAI and soil moisture on CARI for LCar retrieval. Canopy simulation results in **Figure 5a** showed that the overall correlation between CARI and LCar was high ($R^2 = 0.675$, RMSE = 0.675 μg/cm²); however, the correlation differed when LAI values varied. The relationship between CARI and LCar was the worst ($R^2 = 0.455$, RMSE = 0.455 μg/cm²) when LAI values were around 1, which suggests that when LAI values were small, CARI was not sensitive to LCar variations. Indeed, when LAI values were around 1, the information obtained by the canopy spectrum was mostly related to soil background, thus it affected the estimation of LCar. The influence of soil moisture parameter on LCar retrieval with CARI was then investigated when LAI values were 1. Results in **Figure 6** suggested that variations of soil moisture parameter did affect the correlation between CARI and LCar. When the value of soil moisture parameter was 1 (i.e., simulated dry soil), CARI correlated worst with LCar ($R^2 = 0.614$, RMSE = 0.614 μg/cm²). When its value was 0 (i.e., simulated wet soil), CARI showed the best correlation with LCar ($R^2 = 0.922$, RMSE = 1.398 μg/cm²). In general, with the increase of LAI values, the correlation between CARI and LCar increased, and when LAI exceeded 4, the correlation reached 0.89 and remained unchanged. When the LAI values exceeded 4, the fitting equations between CARI and LCar hardly changed, suggesting that when LAI values were larger than 4, CARI might be less sensitive to LCar variations based on canopy spectral data.

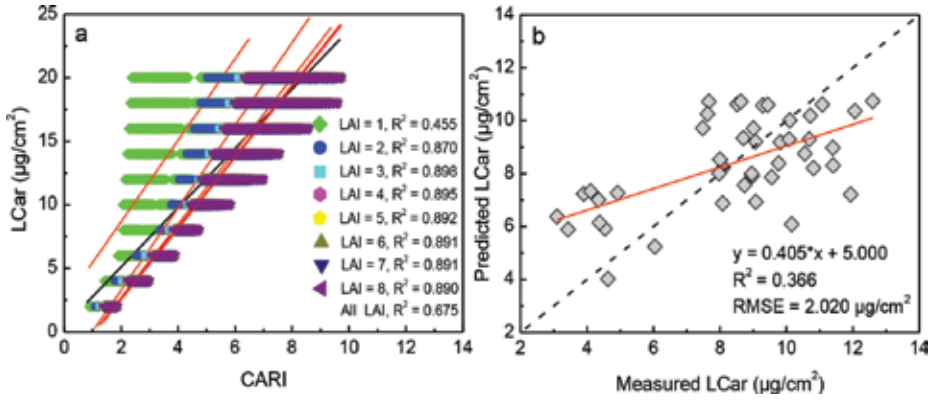

Figure 5. (a) Correlation between CARI and LCar at different LAI values, from all canopy simulations with 4SAIL model (n = 40,800). (b) Scatterplots of measured LCar versus predicted LCar for CARI with canopy reflectance obtained from field data (n = 44). Dashed lines indicate 1:1 lines.

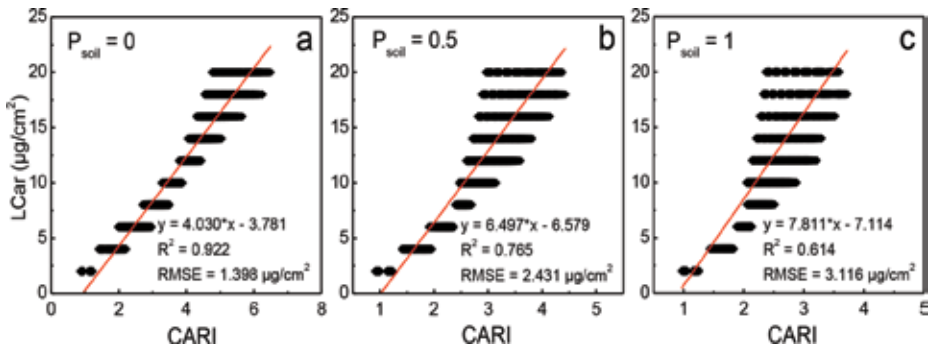

Figure 6. Relationships between CARI and LCar using canopy reflectance simulations with LAI value fixed to 1 at different soil moisture levels. P_{soil} value set as (a) 0, (b) 0.5 and (c) 1. All other parameters for 4SAIL were fixed based on **Table 3** (n = 1700).

Based on the canopy level measured data of winter wheat, LCar estimation results with CARI is shown in **Figure 5b**. Compared to the results that used leaf level data, the estimation accuracy was rather low for LCar retrieval with canopy level spectrum ($R^2 = 0.366$, RMSE = 0.366 μg/cm²), and LCar values lower than 5 μg/cm² were obviously overestimated (**Figure 5b**). However, it should be noted that these low LCar samples were collected at the wheat kernel milk stage, when leaves were close to senescence and LAI values were less than 1. The inaccurate estimation of these low LCar samples would confirm to use caution in the assessment of LCar from CARI, using canopy reflectance, when LAI values are low.

3.5. Discussion

The spectral absorption features of carotenoids in the visible range make it possible for analysis of nondestructive estimation of leaf carotenoids content. However, the overlaps of spectral absorption characteristics of carotenoids and chlorophylls in the visible band making

it challenging to assess LCar with its own absorption features [16]. Based on the reviewed studies on LCar estimation with remote sensing techniques, this chapter established a new carotenoid index (CARI) based on the spectral absorption features of carotenoids. Abundant synthetic data simulated from leaf and canopy models, and measured dataset, including the ANGERS and winter wheat data, were then used to comprehensively investigate its capability in LCar assessment. CARI was established in the form of chlorophyll indices, that is, $CI_{red\text{-}edge}$ and CI_{green}. These chlorophyll indices proposed by Gitelson et al. [15] utilized red-edge (or green) band that was sensitive to chlorophyll variation. Meanwhile, a near infrared waveband was also considered to eliminate the effect of other pigments and backward scattering effect. Many studies had shown that $CI_{red\text{-}edge}$ and CI_{green} can be used to estimate leaf chlorophylls content accurately [34, 35]. Through analyzing the correlation between LCar and reflectance in the visible range from 400 to 800 nm wavelength, we utilized the reflectance of 521 nm band to establish the CARI index. The 521 nm waveband was located in the spectral absorption band of carotenoids and was significantly related to LCar. However, strong correlation between reflectance of 521 nm waveband and LChl existed. In order to eliminate the effect of chlorophylls on carotenoids retrieval, 720 nm waveband was also used in CARI; owing to that, the reflectance of 720 nm band was the most correlated with LChl. With PROSPECT-5 simulations, the new CARI showed a significantly strong linear relationship with LCar. Moreover, CARI showed low correlation with LChl ($R^2 = 0.315$), showing that it was less sensitive to LChl variations and the use of 720 nm band obviously decreased the effect of LChl on LCar estimation to some extent. In addition, CARI showed good estimation of LCar with both the ANGERS data and leaf level experimental data of winter wheat, indicating that it was accurate and robust for LCar assessment with CARI.

With different foliar datasets (leaf simulations, the ANGERS data and winter wheat experimental data), performance of published spectral indices in LCar estimation varied. Carotenoids index, including CRI_{550}, CRI_{700}, $CAR_{red\text{-}edge}$ and CAR_{green}, showed significant linear relationship with LCar with foliar simulated data. However, those indices exhibited poor results for LCar estimation with the ANGERS data and experimental data of winter wheat, suggesting that the accuracy and robustness of these indices in LCar estimation needed to be improved. Compared with CRI_{550} and CRI_{700}, the estimation accuracy in LCar retrieval with $CAR_{red\text{-}edge}$ and CAR_{green} slightly improved. This suggested that adding of a near infrared band (770 nm) in CRI_{550} and CRI_{700} could improve the estimation accuracy [15]. Based on foliar measured data, RARSc showed accurate estimation of LCar (ranking third with the ANGERS data, while ranking second with winter wheat data). These results were consistent with previous studies using RARSc to estimate LCar with measured data [36, 37], suggesting that RARSc was robust in LCar estimation. Performance of RBRI in LCar estimation with simulated and measured data significantly varied: it showed low correlation with LCar in foliar simulations, best results in LCar retrieval with the ANGERS data and poor results with winter wheat experimental data. Based on the spectral absorption features of chlorophylls, Datt [13] proposed the RBRI spectral index, which was used for chlorophylls and carotenoids retrieval. In his study, chlorophylls and carotenoids were significantly correlated. However, in the foliar simulations, their correlation was low ($R^2 = 0.235$), although LChl was significantly related with RBRI ($R^2 = 0.847$). This could explain the correlation between RBRI and

LCar in simulations. In ANGERS data, LCar and LChl were significantly linear correlated (R^2 = 0.908) and RBRI showed strong relationship with LChl (R^2 = 0.785); therefore, RBRI showed high estimation accuracy in LCar retrieval. The RBRI was established on the equation $R_{672}/ (R_{550} \times R_{708})$, which was different from the normalization and ratio form that most indices adopted. The form of the denominator ($R_{550} \times R_{708}$) might help to increase the numerical range of RBRI, making it more sensitive to large values of LCar. However, RBRI might not be sensitive to low values of LCar (<3 μg/cm^2), as samples with LCar values lower than <3 μg/cm^2 were obviously overestimated. In the experimental data of winter wheat, although LCar and LChl were significantly linear related (R^2 = 0.888), RBRI showed poor estimation results for LCar retrieval. This suggested that RBRI might not be stable when used in various datasets for LCar estimation.

Blackburn [14] pointed out that the overlaps between spectral absorption features of carotenoids and chlorophylls might affect the relationship between LCar and PSNDc (or PSSRc). Moreover, 470 nm waveband was used in these spectral indices, which was not the best absorption band for carotenoids. Their performance with the leaf-simulated data and the ANGERS data supported this viewpoint. However, PSNDc and PSSRc showed rather good estimation accuracy in LCar retrieval with winter wheat data. This may be due to the fact that LCar values of the measured foliar data of winter wheat were in the range of 4–12 μg/cm^2. Unlike the ANGERS data numerical range, these indices may be more sensitive to LCar changes in this range. PRI was successfully applied to a variety of studies [38, 39]. In this chapter, PRI showed poor performance in LCar estimation with leaf-simulated data and the ANGERS data. The 531 nm waveband of PRI was used to detect variations of xanthophyll cycle components [31], PRI's relationship with LCar may be overly influenced by a single carotenoid component [40].Compared with the estimated results in simulated data and the ANGERS data, PRI showed the best estimation accuracy in LCar retrieval with winter wheat data. Previous study had also shown that PRI was accurate in LCar estimation in cotton plants [37]. These results indicated that PRI may be suitable for LCar estimation in single-species vegetation. Unlike PRI, PRIm1 showed poor estimation results in all the used datasets. This may be because PRIm1 was devised to reduce the effect of the canopy structure effect and indicated water stress [32]. These results indicated that PRIm1 was not suitable for LCar estimation. Similarly, PSRI was devised to indicate leaf senescence and fruit ripening, it was sensitive to changes of the content ratio of carotenoids to chlorophylls [8]. The assessment results also indicated that PSRI was not suitable for LCar estimation. SRcar showed poor correlation with LCar in simulated data, this was mainly because that the parameters of the leaf-simulated data in this chapter were more complicated than that of Hernandez-Clemente et al. [16]. Similarly, the poor estimation results of LCar with SRcar in the ANGERS data may be related to the diversity of vegetation types. However, SRcar showed good estimation accuracy in LCar assessment with winter wheat data, which indicated that it might be suitable for LCar retrieval with single-species vegetation.

Although the new index CARI showed good estimation results for LCar retrieval with different foliar datasets, the simulation results with canopy level data suggested that CARI was not sensitive to LCar variations when LAI was low (e.g., LAI = 1). Moreover, soil moisture

parameters affected the estimation accuracy of LCar with CARI. When LAI values are low, and soil is in a dry condition, canopy spectral reflectance of plants is mainly controlled by soil reflection; this could weaken plant canopy information, thus reducing LCar estimation accuracy (**Figure 6c**). When the soil is in a wet condition, the overall soil reflectance is lower, thus its confounding effect on LCar estimation seems to be reduced (**Figure 6a**). Our results with measured datasets thus supported the insensitivity of CARI to LCar detection using canopy reflectance when LAI is low. Further investigations on CARI using canopy reflectance acquired with hyper- or multispectral sensors (such as Sentinel-2), are still needed to achieve accurate and robust LCar calibrations, thus providing a promising new tool for assessing information on plant physiological status at the regional scale.

4. Conclusions

This chapter mainly focused on leaf and canopy level radiative transfer model PROSPECT-5 and 4SAIL to simulate abundant leaf and canopy synthetic data and to establish a new carotenoid index (CARI) for LCar assessment based on the spectral absorption features of carotenoids. Abundant measured data, including the ANGERS data and experimental data of winter wheat, were then used to comprehensively evaluate the capability and robustness of CARI in LCar retrieval. Results showed that CARI correlated best with LCar among all the selected spectral indices with leaf-simulated data. Moreover, CARI showed accurate and robust estimation results of LCar with the ANGERS data and experimental data of winter wheat. Further investigation of CARI in LCar retrieval with simulated and measured canopy level data showed that CARI was insensitive to LCar variations when LAI values were low. In these conditions, soil moisture parameters affected the estimation accuracy of LCar with CARI. Overall, we suggest that CARI is suitable for LCar assessment, which could provide basis for LCar nondestructive estimation with remote sensing techniques.

Acknowledgements

The content in this chapter was supported by the National Key Research and Development Program of China (2016YFD0300601) and the National Natural Science Foundation of China (41571354, 41501468, and 41301389) and the Innovation Foundation of Director of Institute of Remote Sensing and Digital Earth, Chinese Academy of Sciences, China (Grant No. Y6XS560030, Y5ZZ01101B). The authors would like to thank those who helped with the field campaign at the National Experimental Station for Precision Agriculture.

Conflict of interest

The authors declare no conflict of interest.

Author details

Wenjiang Huang[1], Xianfeng Zhou[2]*, Weiping Kong[1] and Huichun Ye[1]

*Address all correspondence to: zhouxianfeng@hdu.edu.cn

1 Key Laboratory of Digital Earth Science, Institute of Remote Sensing and Digital Earth, Chinese Academy of Science, Beijing, China

2 College of Life Information Science and Instrument Engineering, Hangzhou Dianzi University, Hangzhou, China

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Edited by Leila Queiroz Zepka, Eduardo Jacob-Lopes and Veridiana Vera De Rosso

The book "Progress in Carotenoid Research" presents an authoritative and comprehensive overview of the biology, biochemistry, and chemistry of carotenoids. Divided into 14 discrete parts, this book covers topics on basic science and applied technology of carotenoid molecules. This book provides an insight into future developments in each field and has an extensive bibliography. It will be an essential resource for researchers and academic and industry professionals in the natural pigment field.

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ISBN 978-1-83881-752-7