EXTRACTION, STABILITY AND ANTIOXIDANT ACTIVITIES OF LUTEIN FROM YELLOW SILK COCOONS OF SILKWORM (*Bombyx mori*)

Potchanee Kaewkumsan

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การสกัด ความคงตัว และฤทธิ์ต้านออกซิเดชันของลูทีนจากรังไหมเหลืองของหนอนไหม (*Bombyx mori*)

นางสาวพจนีย์ แก้วคำแสน

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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EXTRACTION, STABILITY AND ANTIOXIDANT ACTIVITIES OF LUTEIN FROM YELLOW SILK COCOONS OF SILKWORM (*Bombyx mori*)

Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for a Master's Degree.

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พจนีย์ แก้วคำแสน : การสกัด ความคงตัว และฤทธิ์ต้านออกซิเดชันของลูทีนจากรังไหมเหลืองของหนอนไหม (*Bombyx mori*) (EXTRACTION, STABILITY AND ANTIOXIDANT ACTIVITIES OF LUTEIN FROM YELLOW SILK COCOONS OF SILKWORM (*Bombyx mori*)) อาจารย์ที่ปรึกษา : ผู้ช่วยศาสตราจารย์ ดร.มาโนชญ์ สุธีรวัฒนานนท์, 137 หน้า.

ลูทีนและสเตอริโอไอโซเมอร์ซีแซนทีนเป็นแคโรทีนอยด์เพียงสองชนิดเท่านั้นที่ถูกบ่งชี้ว่าพบสะสมอยู่ในจุดด่างที่มีสีเหลืองบริเวณศูนย์กลางรับภาพของเรตินามนุษย์ ซึ่งเชื่อว่ามีบทบาทสำคัญในการกรองแสงสีน้ำเงินที่มีระดับพลังงานสูงและเป็นสารต้านออกซิเดชันที่มีประสิทธิภาพในการลดระดับพลังงานและดักจับ reactive oxygen species และป้องกันการเกิดโรคจอประสาทตาเสื่อมและต้อเนื้อ ต้อกระจกได้ รังไหมเหลืองถือเป็นวัตถุดิบแหล่งใหม่ที่ใช้ในการผลิตลูทีนเนื่องจากให้ปริมาณผลผลิตและความบริสุทธ์ของลูทีนสูง การใช้ประโยชน์จากกระบวนการ degumming ด้วยความร้อนร่วมกับการสกัดด้วยตัวทำละลายอินทรีย์ให้ผลผลิตของลูทีนจากรังไหมเหลืองประมาณร้อยละ 0.052 โดยน้ำหนักแห้ง (all-*E*)-lutein ถูกตรวจพิสูจน์ด้วย C_{30}-reverse phase HPLC column ว่าเป็นรงควัตถุหลักในรังไหมเหลืองร่วมกับซีแซนทีนและ (*Z*)-geometric isomers ซึ่งมีอยู่ในปริมาณเล็กน้อย สารละลายที่ได้จากการ degumming เป็นผลผลิตอีกชนิดหนึ่งจากกระบวนการสกัดรงควัตถุประกอบด้วยโปรตีนไหมซิริซินเป็นองค์ประกอบหลักที่มีขนาดน้ำหนักโมเลกุลแตกต่างกันขึ้นอยู่กับสภาวะการให้ความร้อน การ degumming ที่ 105 และ 120 องศาเซลเซียส จะทำให้ได้สารประกอบเชิงซ้อนลูทีน-ซิริซินจากสารละลายที่ได้จากการ degumming การศึกษาลักษณะของสารประกอบเชิงซ้อนลูทีน-ซิริซินโดยใช้ anion-exchange chromatography, gel filtration และ RP-HPLC แสดงให้เห็นว่า ลูทีนส่วนใหญ่พบอยู่ในซิริซินที่มีขนาดโมเลกุลต่ำ (<43 kDa) ผลจากการทดสอบความคงตัวแสดงให้เห็นว่า สารสกัดลูทีนมีความไวต่อแสงและความร้อน การสลายตัวของลูทีนจากความร้อนเป็นไปตามจลศาสตร์ของปฏิกิริยาอันดับหนึ่งและมีความสอดคล้องกับจลศาสตร์ของปฏิกิริยาอันดับหนึ่งแบบมีการเปลี่ยนแปลงเป็นสองช่วง สามารถเพิ่มความคงตัวและยืดอายุการเก็บของลูทีนได้เมื่อเก็บรักษาในสภาวะที่เหมาะสม กิจกรรมการเป็นสารต้านออกซิเดชันแสดงเป็นปริมาณของลูทีนที่สามารถลดความเข้มข้นเริ่มต้นของ $DPPH^{\cdot}$ ลงได้ร้อยละ 50 (EC_{50}) มีค่าเท่ากับ 3.62 ± 0.02 mol of lutein mol^{-1} $DPPH^{\cdot}$ สารสกัดลูทีนจากรังไหมเหลืองแสดงกิจกรรมการเป็นสารต้านออกซิเดชันที่มีประสิทธิภาพสูงเมื่อศึกษาด้วยวิธี ferric reducing antioxidant power (FRAP).

สาขาวิชาเทคโนโลยีอาหาร ลายมือชื่อนักศึกษา_____

ปีการศึกษา 2552 ลายมือชื่ออาจารย์ที่ปรึกษา_____

POTCHANEE KAEWKUMSAN : EXTRACTION, STABILITY AND ANTIOXIDANT ACTIVITIES OF LUTEIN FROM YELLOW SILK COCOONS OF SILKWORM (*Bombyx mori*). THESIS ADVISOR : ASST. PROF. MANOTE SUTHEERAWATTANANONDA, Ph.D., 137 PP.

LUTEIN/YELLOW SILK COCOONS/*Bombyx mori*/SERICIN/EXTRACTION/ STABILITY/ANTIOXIDANT ACTIVITIES

Lutein and its stereoisomer zeaxanthin have been identified as the only two carotenoids deposited in the macular pigment of human retina. They are believed to play a major role as a filter of high energy blue light, and a potent antioxidant that quenches and scavenges photo-induced reactive oxygen species and prevents the pathogenesis of age-related macular degeneration (AMD) and cataracts. Silk cocoons are a novel source of raw material for lutein production due to their high yield and purity of lutein. Through the use of heat degumming process together with organic solvent extraction, yellow silk cocoons yield approximately 0.052% of lutein in a dry basis. The (all-*E*)-lutein was identified using the C_{30}-reverse phase HPLC column to be a major pigment in the silk cocoons with small presence of zeaxanthin and (*Z*)-geometric isomers. The degumming solutions, another product from the pigment extraction process, contained the predominant silk protein sericin with different apparent molecular mass depending on the heat treatment conditions. Degumming at 150 and 121ºC, the lutein-sericin complex can be obtained from degumming solutions. The characterization of the lutein-protein complex using anion-exchange chromatography, gel filtration, and RP-HPLC revealed the majority of lutein with low molecular mass sericin (<43 kDa). Results from the stability test suggest that lutein

extract be susceptible to lights and heat. Thermal degradation of lutein followed the first-order reaction kinetic and correlated well with two-stage first-order kinetic. Its stability and shelf-life can be extended when stored at appropriate conditions. The antioxidant activity expressed as the amount of lutein able to reduce the initial $DPPH^{\cdot}$ concentration to 50% (EC_{50}) was 3.62 ± 0.02 mol of lutein mol^{-1} $DPPH^{\cdot}$. Lutein extracted from silk cocoons showed a powerful antioxidant activity as determined by the ferric reducing antioxidant power (FRAP) method.

School of Food Technology Student's Signature _____

Academic Year 2009 Advisor's Signature _____

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CONTENTS

Page

CONTENTS (Continued)

Page

CONTENTS (Continued)

Page

CONTENTS (Continued)

Page

CONTENTS (Continued)

Page

LIST OF TABLES

Table **Page**

LIST OF TABLES (Continued)

Table **Page**

LIST OF FIGURES

Table **Page**

LIST OF FIGURES (Continued)

Table **Page**

LIST OF FIGURES (Continued)

Table **Page**

LIST OF ABBREVIATIONS

ANOVA	=	Analysis of variance
α	=	Alfa
γ	=	Gamma
β	=	Beta
^{o}C	=	Degree Celsius
DEAE	=	Diethylaminoethyl cellulose
et al.	=	et alia (and others)
Fe^{2+}	=	Ferrous
Fe^{3+}	=	Ferric
$Fe(TPTZ)^{2+}$	=	Fe^{2+} complex of *2*,4,6-Tris(*2*-pyridyl)-1,3,5-triazine (TPTZ)
$Fe(TPTZ)^{3+}$	=	Fe^{3+} complex of *2*,4,6-Tris(*2*-pyridyl)-1,3,5-triazine (TPTZ)
g	=	Gram
g^{-1}	=	Reciprocal gram
$\times g$	=	Relative centrifuge force
h^{-1}	=	Reciprocal hour
kDa	=	Kilodalton
kJ mol^{-1}	=	Kilojoules/mol
λ_{max}	=	Maximum wave length
L	=	Litter
L^{-1}	=	Reciprocal litter
Lux	=	SI unit of illuminance and luminous emittance

LIST OF ABBREVIATIONS (Continued)

M	=	Molar
mAU	=	Miliabsorbance unit
mg	=	Milligram
min	=	Minute
mL	=	Milliliter
mm	=	Millimeter
mM	=	Millimolar
mM kg^{-1}	=	Milimolar/kg
mM L^{-1}	=	Milimolar/litter
µL	=	Microlitter
µmol L^{-1}	=	Micromolar/litter
nm	=	Nanometer
%	=	Percentage
Rpm	=	Revolution per minute
SAS	=	Statistical analysis system
SDS-PAGE	=	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
V	=	Voltage
wt.	=	Weight
ζ	=	Zeta

CHAPTER I

INTRODUCTION

1.1 Introduction

The mulberry silkworm *Bombyx mori* has been commercially domesticated for centuries, mostly in temperate and tropical regions. In Thailand, sericulture is a famous sideline activity along with agriculture and thereafter becomes a major trade due to an increasing in economical demand of unique characteristic silk products. The races of mulberry silkworm, *B. mori*, as well as non-mulberry or wild silkworm are classified into several groups based on number of larval moults, number of broods per year or voltinism, place of origin, color of cocoons, place of rearing, and so on (Ganga, 2003). Based on voltinism, three kinds of races are recognized in mulberry silkworm including univoltines, bivoltines, and multivoltines that withstand environmental fluctuations differently. For multivoltines, they are specific for tropical areas whereas univoltine and bivoltines are for temperate regions. Their morphological characters are resistant to diseases and tolerant environmental fluctuations (Kaplan et al., 1994). Thus, multivoltinies or polyvoltinies, mostly produce yellow cocoons, are widely reared in several regions of tropical countries including Thailand. However, the yellow cocoons are small in size and poor in commercial quality. Small size of cocoons limits the amount and length of silk yarn and filament. In these cases, bivoltinie races from Japan and China origins which generally produce large white cocoons are more advantage.

Like other silkworm races, raw silk reeling from domesticated *B. mori*, consists of filaments of protein called fibroin held together by a gum-like protein termed sericin (Seves et al., 1998). Prior to weaving, raw silk is usually degummed by removal of most sericin, resulting in shiny silk thread, soft handle, and elegant drape highly appreciated by consumers. Nevertheless, because yellow cocoons are composed of a large amount of sericin, consequently, degumming process required large amount of water and energy (Fabiani et al., 1996). In addition, wastewater from yellow silk production consequently contains high content of sericin, resulting in high cost waste water treatement. Thus, of the results, used of yellow cocoons is decreased in textile industry. These evidences show that wastewater is hardly treated, particularly, by many household manufacturers in Thailand. Eventually, the value of yellow cocoons dramatically decreases in current silk market.

However, recent studies have shown that the unique functional properties of wastewater constituents like sericin and lutein. Sericin is known to have biologically functional properties as antioxidant activity (Kato et al., 1998), reduction of UV-B induced damage (Zhaorigetu et al., 2003), suppression of colon tumors (Zhaorigetu et al., 2001), and enhancement of the intestinal absorption of several minerals (Sasaki, Yamada and Kato, 2000). Additionally, sericin has a skin moisturizing effect, possibly due to high content of serine, and anti-wrinkle action (Kato et al., 1998). These unique characteristics of sericin make this protein a valuable natural ingredient for cosmetic and food industries. On the other hand, recent studies have demonstrated the unique bioavailability of lutein to protect the common eye diseases of aging, cataract and aged-related macular degeneration (AMD), the major cause of blindness in elder (Alves-Rodrigures and Shao, 2004; Dachler, Kohler and Albert, 1998; Olmedilla, et al., 2003). As potent antioxidant activity, lutein is also directly involved with the

prevention of certain-type of cancer (Deli et al., 2003) and the risk reduction of cardiovascular diseases (Tsuchida et al., 2004; Alves-Rodrigures and Shao, 2004). Nevertheless, the extraction and characterization of lutein for commercial benefits are mostly limited to plant sources. Therefore, the study of lutein found in domesticated *B. mori* silk cocoons is very important.

In Thailand, the production of lutein in commercial scale is currently not available. Lutein as well as sericin is very expensive and mostly imported from abroad. By recovery both sericin and yellow pigments, particularly lutein, from silk industrial wastewater or even separation them from silk production processes with proper condition is therefore an alternative way to increase the value of domesticated *B. mori* silk cocoons.

1.2 Research objectives

The objectives of this research were:

(1) To extract of lutein from yellow silk cocoons of silkworm (*B. mori*),

(2) To identify and quantify of lutein found in the yellow silk cocoons,

(3) To study the stability of lutein in lutein extract and sericin-lutein complex,

(4) To study antioxidant activities of extracted lutein as free radical-scavenging ability and ferric reducing antioxidant power,

(5) To characterize sericin-lutein complex in degumming solution.

1.3 Research hypotheses

Lutein is believed to function as a filter of high energy blue light and a potent antioxidant. Presence of lutein in skin and oral consumption may serve to protect skin

from UV-induced damage and reduce the risk of eye diseases such as aged-related macular degradation (AMD) and cataracts. Lutein from yellow silk cocoons of silkworm (*B. mori*) which possibly co-exist with silk protein sericin, may serve such functional properties and greater when comparing to that from plant extract.

1.4 Expected results

The purified lutein from yellow silk cocoons of silkworm (*B. mori*) would be obtained and identified. Lutein extract from the yellow silk cocoons may act as effective antioxidant. Sericin-lutein complex may enhance the stability of lutein that is appropriate for commercial use as an active ingredient for food supplements, pharmaceutical, and cosmetics products.

CHAPTER II

LITERATURE REVIEWS

2.1 Lutein

2.1.1 Lutein and human health

Lutein [(3R,3'R,6'R)-β,ε-carotene-3,3'-diol] is a number of xanthophylls, a family of carotenoids, with non-provitamin A activity (Rodriguez-Amaya, 2001). It is a type of fat-soluble yellowish pigment mostly found in higher plants, algae and photosynthetic bacteria (Feltl et al., 2005). Because lutein as well as other carotenoids can only be biosynthesized by plants and microorganisms, their presence in animals is attributed to ingestion via foods and accumulation in certain tissues (Deli et al., 2003). Lutein and its isomer zeaxanthin have been identified as the only carotenoids present in specific eye tissues (Aman et al., 2005). They are highly concentrated in the macular fovea, a small area of the retina responsible for central vision and high visual acuity. In humans, as in plants, lutein is believed to function as a filter of high energy blue light, and an antioxidant that quenches and scavenges photo induced reactive oxygen species (ROS) (Alves-Rodrigures and Shao, 2004) which are highly reactive and can damage DNA and lipids. On the other hand, evidences have been proven that lutein consumption is inversely related to eye diseases such as aged-related macular degradation (AMD), the leading cause of blindness among the elderly, and cataracts (Alves-Rodrigures and Shao, 2004; Dachler, Kohler and Albert, 1998; Olmedilla, et al., 2003). High dietary intakes and high blood levels of lutein can reduce the risk of

AMD and cataracts, the major causes of visual impairment. Figure 2.1 shows the proposed model of AMD protection by lutein and its isomer. Besides the bioavailability as potent antioxidant, recent studies suggest that xanthophylls, especially lutein, be directly implicated certain-type cancer prevention (Deli et al., 2003). Presence of lutein in skin and oral consumption may also serve to protect skin from UV-induced damage and may reduce the risk of cardiovascular diseases (Tsuchida et al., 2004; Alves-Rodrigures and Shao, 2004).

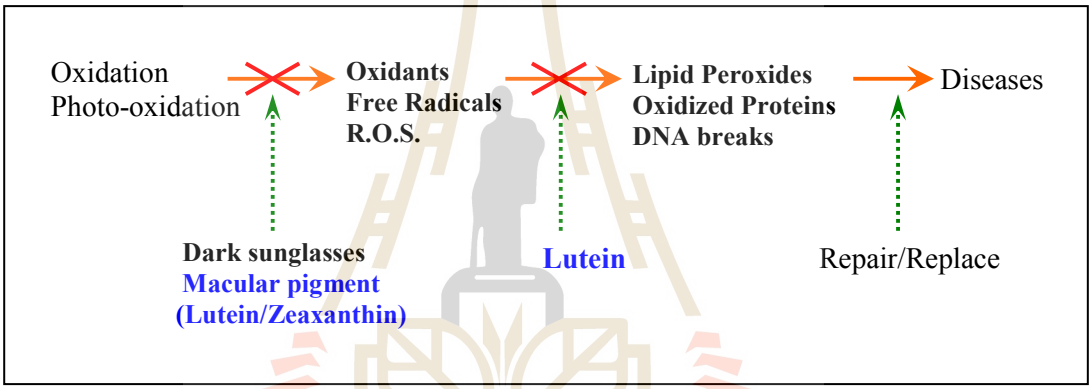

Figure 2.1 Proposed model for AMD protection by lutein and zeaxanthin.

From: Winkler et al. (1999)

Although, lutein can be found in human diets with most abundantly in dark, leafy green such as spinach and kale, and some foods with yellow color, such as corn and egg yolk, the average daily intake of lutein as observed in developed countries like US and Europe (Alves-Rodrigures and Shao, 2004) is not enough to reduce the risk of eye diseases as well as other related symptoms. In such regions, mean daily intake of lutein, combined with zeaxanthin, varies from 0.8 to 4 mg per day, and lutein alone averages for 2.45 mg per day (Beatty et al., 2004), whereas the 57% lower

AMD risk needs for 6 mg per day (Alves-Rodrigures and Shao, 2004). These suggest that the average daily intake of lutein, nowadays, not be enough to reduce the risk of AMD. Moreover, humans can carry out only limited metabolic transformations of carotenoids that carotenes and xanthophylls can not be converted to be each other (Yemelyanov, Katz and Bernstein, 2001), suggesting that the sufficient consumption of specific dietary carotenoids be certainly needed. Therefore, it is not surprising why lutein is potential used in dietary supplement as well as in certain foods and beverages, and increase of distinctly interest in food market nowadays.

2.1.2 Biosynthesis and natural existing of lutein

Lutein is a carotenoid pigment synthesized by photosynthetic microorganisms and plants but not by animals. In general, as in most food carotenoids, lutein is primarily biosyntherzied from the condensation of two C_{20} geranyl geranyl diphosphate molecules, or GGPP, passing the first C_{40} carotenoid phytoene (DellaPenna, 1999; Hirschberg et al., 1997). Phytoene, consisting 3 conjugated double bonds, is sequentially desaturated to lycopene with 11 conjugated double bonds and, then, converted to be lutein through the cyclization of both ends of the molecule followed by hydroxylation at 3-, and 3' position (Figure 2) (Rodriguez-Amaya, 2001).

Because of a conjugated system in which the π-electrons are effectively delocalized over the entire length of the polyene chain, this characteristic is responsible for molecular shape, chemical reactivity, light absorbing properties, and color of lutein as well as other carotenoids (Dutta, Chaudhuri and Chakraborty, 2005). However, the presence of two hydroxyl group attached to both ends of *β*-ionone rings in the molecule of xanthophylls, like lutein and zeaxanthin (Figure 2.2), makes them more hydrophilic than other carotenoids found in blood and tissues such as

hydrocarbon carotenoids; α-carotene, β-carotene, and lycopene. These are believed to impart xanthophylls playing a critical role in their biological function differently from others.

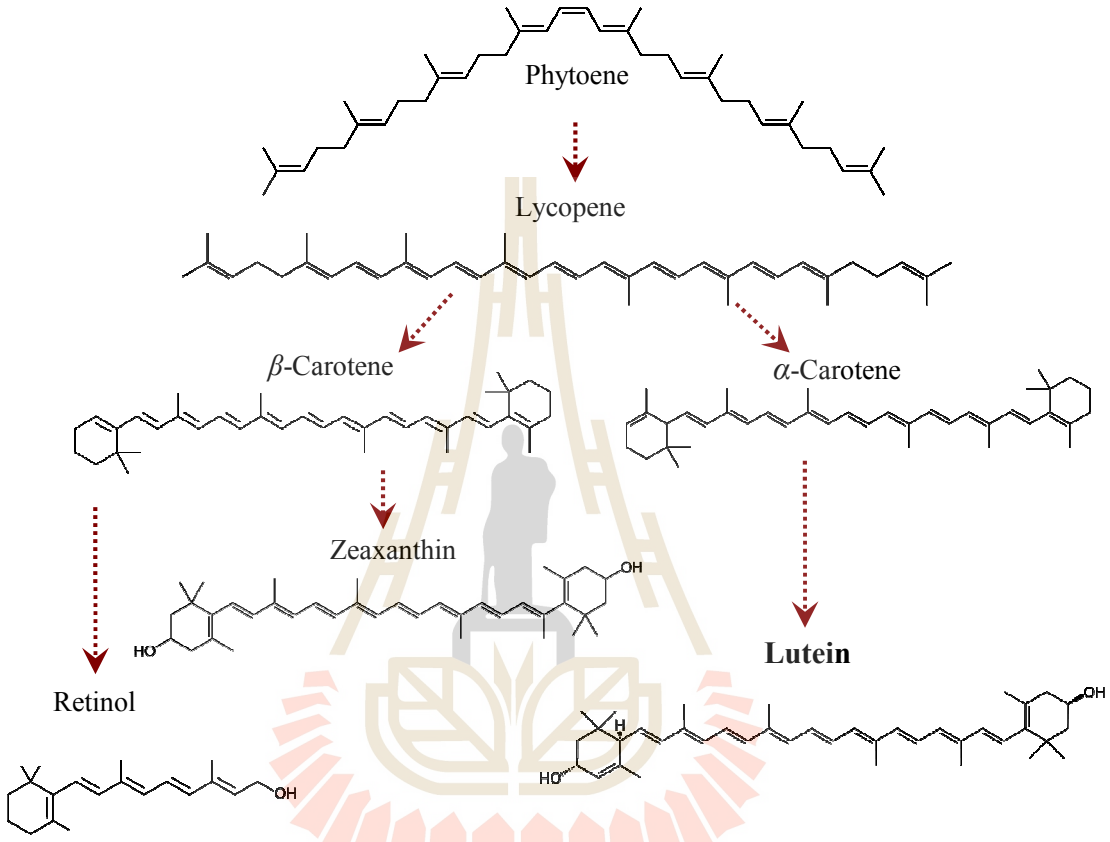

Figure 2.2 Diagram of lutein pathway.

Lutein is a lipophilic molecule and generally insoluble in water. The molecular formula of lutein is $C_{40}H_{56}O_2$ and its molecular weight is 568.88 Daltons. Owing to its three chiral centers, there are 8 stereoisomers of lutein. While, zeaxanthin has two chiral centers and therefore, has four isomeric forms. However, in contrast to lutein, zeaxanthin is a symmetric molecule and (3R, 3'S)- and (3S, 3'R)-stereoisomers are identical. Therefore, zeaxanthin has only three stereoisomeric forms and its (3R, 3'S)- or (3S, 3'R)- stereoisomer is called *meso*-zeaxanthin (Figure 2.3).

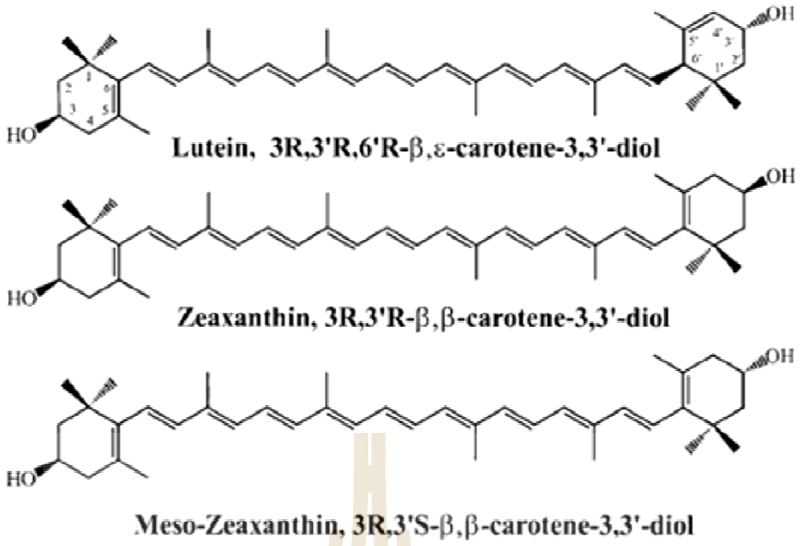

Figure 2.3 The structure of major carotenoid components found in the human macula. From: Landrum and Bone (2001)

Over 600 carotenoids have been described in nature (Furr and Cark, 1997); lutein, zeaxanthin, and *meso*-zeaxanthin are known as only three carotenoids consisting in human macular like macular pigments (Landrum and Bone, 2001). Although all carotenoids in human are exclusively derived from the diet, except in the macular, neither human diet nor plasma includes *meso*-zeaxanthin. It has been proposed that lutein is most likely isomerized to the nondietary *meso*-zeaxanthin which probably has a greater ability to quench oxygen radicals, in the retina via the migration of a double bond. This may explain by the difference between the 3:1 ratio of lutein to zeaxanthin in blood and the 1:2 ratio in fovea. In addition, a mapping of ratios of *meso*-zeaxanthin to zeaxanthin versus lutein to zeaxanthin as a function of increasing distance from the fovea suggests that a conversion mechanism is concentrated in the macula (Ahmed, Lott and Marcus, 2005). Corresponding to the distribution of xanthophylls in human diet, especially in dark green leafy vegetables,

there is 15-47% of lutein, but a very low content about 0-3% of zeaxanthin (Sommerburg et al., 1998). The latter investigation also supports that there are an approximately 7-fold of lutein higher than zeaxanthin in human diet (Beatty et al., 2004), whereas, human plasma contains higher amount of zeaxanthin than existing in diet, and up to 2% of nondietary *meso*-zeaxanthin (Khachik et al, 2002). However, the nature of the relationships between lutein and zeaxanthin in foodstuffs, blood, and macula is confounded by many variables including processes which influence digestion, absorption, and transport of the compounds in question, and the accumulation and stabilization of the carotenoids in the tissues. A clear understanding of the specific uptake and stabilization mechanisms of lutein and zeaxanthin is, therefore, advantageous.

There are around 50 carotenoids commonly found in fruits and vegetables consumed by humans. Notably, most of these are geometrical isomers and metabolites of a few parent carotenoids (Deming and Erdman, 1999). Table 2.1 shows sources of lutein and its isomer zeaxanthin which mostly occurred in dark green vegetables such as kale, parsley, spinach, collards, broccoli, and green peas, and yellow-orange fruits and vegetables such as squash, oranges, prunes, and peaches. Food with yellow color like fortified egg yolks also serves a good dietary source of lutein (Alves-Rodrigures and Shao, 2004; Handelman et al., 1999; Schlatterer and Breithaupt, 2006). Besides, these xanthophylls are found in yellow-red fruits and vegetables, such as apricots, carrots, cantaloupe and tomatoes, but lower than β-carotenes (Furr and Cark, 1997). Lutein, like other carotenoids, is particularly concentrated in chromoplasts or chloroplasts of plant foods and is non-covalently bound to protein or fiber, dissolved in oils or exists in crystalline form. In yellow-orange fruits and vegetables as well as

Table 2.1 Quantitative distribution of lutein, zeaxanthin, and related geometrical isomers in selected fruits and vegetables[a].

Foods	Concentration of lutein and zeaxanthin (μg/100g)										
	Lutein (L)						Zeaxanthin (Z)				L/Z
	(all-*E*)	(9-*Z*)	(9'-*Z*)	(13-*Z*) + (13'-*Z*)	(13-*Z*), (13'-*Z*)	total *E* + *Z*	(9-*Z*)	(9'-*Z*)	(13-*Z*)	total *E* + *Z*	ratio
Greens											
Beans, green	390.0	19.5	5.8	1.6	1.2	418.1	23.0	12.0	*b*	35.0	12
Beans, lima (canned)	275.5	27.5	27.5	19.6	6.0	356.1	16.0	*b*	*b*	16.0	22
Broccoli	1343.0	65.0	16.4	81.7	4.5	1510.6	9.4	33.4	*b*	42.8	35
Collards	4940.0	72.0	77.0	11.0	20.0	5120.0	128.0	12.0	*b*	140.0	37
Kale	13053.0	390.0	815.0	678.0	64.0	15000.0	50.2	189.8	*b*	240.0	63
Lettuce, romaine[c]	148.0	12.0	3.0	5.0	2.0	170.0	2.5	5.5	*b*	8.0	21
Parsley	9924.0	351.7	53.1	481.2	10.0	10820.0	134.0	368.0	*b*	502.0	22
Peas (canned)	661.8	21.1	8.0	26.1	2.0	719.0	40.1	10.9	*b*	51.0	14
Spinach	8447.0	223.7	38.0	442.3	6.0	9157.0	130.8	394.3	*b*	525.1	17
Yellow-orange									*b*		
Corn (canned)	163.4	21.0	3.0	10.2	*b*	198.0	310.0	22.7	*b*	332.7	0.6
Mango	10.0	*b*	*b*	*b*	*b*	10.0	10.0	*b*	*b*	10.0	1.0
Nectarine	12.2	4.5	3.3	*b*	*b*	20.0	62.0	108.0	*b*	170.0	0.1
Oranges	350.0	*b*	*b*	*b*	*b*	350.0	250.0	*b*	*b*	250.0	1.4
Oranges, mandarine	48.3	14.2	2.0	6.0	*b*	70.5	52.0	8.0	*b*	60.0	1.2
Papaya	23.1	*b*	*b*	*b*	*b*	22.1	22.1	*b*	*b*	22.1	1.0
Peaches	20.0	*b*	*b*	*b*	*b*	20.0	20.0	*b*	*b*	20.0	1.0
Plum, red	40.0	*b*	*b*	*b*	*b*	*b*	*b*	*b*	*b*	*b*	*b*
Squash, acorn	50.0	*b*	*b*	*b*	*b*	*b*	*b*	*b*	*b*	*b*	*b*
Squash, butternut	1793.0	146.0	334.0	127.0	*b*	280.0	280.0	*b*	*b*	280.0	8.6

Note: [a] With the exception of the canned foods, all fruits and vegetables were analyzed in the raw form. The detection limit for HPLC analysis of carotenoids was 0.1 ng. [b] Not detected. [c] Romaine lettuce also contained lactucaxanthin, 148 μg/100 g of edible food. *E*/Z, *cis- trans* forms.

From: Humphries and Khachik (2003)

egg yolk and marigold flower, lutein is usually esterified with various long-chain fatty acids such as lauric, mystric and palmitic acids (Breithaupt and Bamedi, 2001; Breithaupt, Wirt and Bamedi, 2002; Hojnik, Škerget and Knez, 2008; Pérez-Gálvez and Mínguez-Mosquera, 2005). Papaya, peaches, prunes and squash all contain lutein diesters, whereas squash and peaches also contain lutein monoesters and zeaxanthin diesters, respectively (Zaripheh and Erdman, 2002). Evidence suggests that the esterification of the hydroxyl group with fatty acid might stabilize lutein against heat and UV-light degradation (Subagio, Wakaki and Morita, 1999). Free lutein is very unstable against heat, whereas lutein monoester is slightly stable and lutein diester is very stable. However, the interaction between lutein and food matrices influencing on the digestion and absorption of lutein has been discussed (Bowen et al., 2002; Zaripheh and Erdman, 2002).

2.1.3 Absorption of lutein and its bioavailability

In general, xanthophylls and another structural classified carotenoid, carotene, are lipid-soluble molecules that follow the absorption pathway of dietary fat. The absorption involves several steps from the breakdown of food matrix and release of carotenoids into the lumen of the gastrointestinal tract through their incorporation into lymphatic lipoproteins (Zaripheh and Erdman, 2002). The efficient digestion and absorption of dietary fat, as well as the presence of bile salt micelles, is essential for carotenoid absorption. Competition among carotenoids and other dietary components for absorption, transport, and uptake by tissues is documented (Dutta, Chaudhuri and Chakraborty, 2005; Zaripheh and Erdman, 2002), but needs further research. From limited data it seems that the more polar carotenoids, xanthophylls, may be absorbed more efficiently than are carotenes, hydrocarbon carotenoids (Furr and Clark, 1997). As results of xanthophylls, particularly lutein and its metabolic products are well

solubilized and preferably incorporated into surface of lipoproteins such as chylomicrons, LDL and HDL. These may enhance the transportation of lutein via blood circulating system and then accumulated in specific tissues, whereas 80-85% of hydrocarbon carotenoids preferably accumulated in the adipose tissues (Deming and Erdman, 1999; Dutta, Chaudhuri and Chakraborty, 2005; Yemelyanov, Katz and Bernstein, 2001). In human serum, only six major carotenoids, particularly lutein and lycopene (Table 2.2) occur with the estimated half life for 11-14 days of lutein, as well as zeaxanthin, lycopene, α-carotene, and β-carotene (Dutta, Chaudhuri and Chakraborty, 2005; Shao, 2001). Tissues differently take up those carotenoids, with lutein and zeaxanthin specifically accumulating in the macula region of the eye and strong associating with the decrease of AMD risk (Shao, 2001; Zaripheh and Erdman, 2002).

Table 2.2 Distribution of the major carotenoids in human serum.

Carotenoid	%Relative distribution in serum
Lutein	20
Lycopene	20
α-Carotene	10
ζ-Carotene	10
Phytofluene	8
β-Cryptoxanthin	8
β-Carotene	6
α-Cryptoxanthin	4
Phytoene	4
Anhydrolutein	3
Zeaxanthin	3
γ-Carotene	2
Neurosporene	2

From: Shao (2001)

Food matrix may affect the optimal absorption difficult to achieve. Moreover, comparing to free lutein, a greater amount of fat is required for the optimal de-esterification of lutein esters. Because ester forms of lutein are more hydrophobic making them more difficult to solubilization. Thus, at least 3 g of fat are required for the sufficient solubilization of lutein esters and whether secretion of esterase and lipases from the pancreas during the digestion and absorption (Dutta, Chaudhuri and Chakraborty, 2005; Zaripheh and Erdman, 2002).

2.2 Yellow silk cocoons of silkworm (*B. mori*)

2.2.1 Yellow silk cocoons: source of lutein and sericin

Yellow silk cocoon is one of some cocoons from many varieties of the silkworm, *B. mori*. Recent studies have been proven that the pigments in yellow or golden-yellow cocoons are derived from carotenoids, whereas the cocoons in others color such as sasa (yellowish green) and green are from flavonoids (Tabunoki et al., 2002; Tabunoki et al., 2004; Tsuchida et al., 2004a). These pigments are absorbed from mulberry leaves, only a food source of the wild-type *B. mori*. They are then transferred from a midgut to silk grand via the hemolymp, and eventually accumulated in the layers of the cocoon sericin (Tabunoki et al., 2004). It is emphasized that among those carotenoids, xanthophylls, principally lutein, have been indicated in previous study as predominant carotenoids in the yellow cocoons (Jouni and Wells, 1996). For silk textiles, these pigments are partially removed from the silk cocoons without any use. Moreover, silk protein like sericin, the second main constituent of silk fibers (20-30% of the total cocoon weight), is also mostly removed from the cocoon during degumming process (Zhaorigetu et al., 2001). The wastewater from such process is,

therefore, composed of both lutein and sericin that is hardly treated by common wastewater management system. However, the unique functional properties of both lutein and sericin have been revealed. These compounds currently benefit for food and cosmetic productions as valuable natural ingredients. Consequently, the isolation and extraction as well as characterization of silkworm, *B. mori*, in a form of lutein-binding protein, have been initially investigated (Jouni and Wells, 1996).

In *B. mori.*, a major fate of absorbed lutein is accumulated in the layers of cocoon sericin, which imparts cocoons to be yellow in color (Jouni and Wells, 1996; Tabunoki et al., 2004; Tsuchida et al., 2004). Except of lutein, sericin itself is known as the silk protein that resists oxidation, has antimicrobial effect, prevents UV-induced damage, and absorbs and releases moisture easily (Zhaorigetu et al., 2003; Teramoto and Miyazawa, 2006). Kato and others (1998) have demonstrated that sericin inhibits the activity of tyrosinase (polyphenol oxidase), which is responsible for the browning reaction of various foods and biosynthesis of skin melanin. Furthermore, Zhaorigetu's research group (2001) has proven that dietary sericin suppresses the development of colon tumors by reducing oxidative stress, cell proliferation, and nitric oxide production. Thus, sericin is widely used as a valuable ingredient of food, cosmetic and medicine. Since, silk cocoons consist two main proteins such as sericin and fibroin, these proteins may play an important role of silk cocoon pigmentation by assembling with lutein as well as other constituting carotenoids in the cocoon shells. Unfortunately, the characterization of lutein in silk cocoons has not been investigated. Therefore, to recover lutein as well as sericin from silk production for further use, the development of appropriate extraction and purification methods is needed.

2.2.2 Identification and characterization of carotenoid-binding protein

Because lutein as well as other carotenoids can only be biosynthesized by plants and microorganisms, their presence in animals is attributed to ingestion via foods and accumulation in certain tissues (Deli et al., 2003). Jouni and Wells (1996) reported that, once absorbed, carotenoids were either irreversibly or reversibly modified. Irreversible modification included the decomposition of carbon skeleton into smaller units or the addition of new functional groups such as hydroxylation. Reversible modification included the esterification of the hydroxyl carotenoids with long chain fatty acids or the conjugation of the carotenoids with proteins forming carotenoid-binding protein complexes, namely carotenoproteins, which are water-soluble and more stable than the carotenoids alone. Differential and selective absorption of different classes of carotenoids suggests the presence of carotenoid-binding proteins that assist in carotenoids absorption. In addition, the presence in variable concentrations and forms of carotenoids in different tissues strengthened the idea that carotenoid-binding proteins may play a role in uptake and accumulation of carotenoids in tissues (Bone et al., 2000; Tsuchida et al., 2004).

In nature, carotenoids are responsible for many of the red, orange, and yellow hues of plant leaves, fruits, and flowers, as well as the colors of some birds, insects, fish, and crustaceans (Stahl and Sies, 2003). Because of their hydrophobic nature, carotenoids in plant and animal cells are found in association with lipid-protein complexes, where they interact with hydrophobic domains. The unique protein-chromophore interactions in these complexes are responsible for the red, blue, and green coloration of carapaces in many invertebrates, such as lobster, shrimp, and crayfish. In humans, over 80% of the carotenoids absorbed from the intestine accumulate in adipose tissues, transported there by lipoproteins (Vishnevetsky, Ovadis

and Vainstein, 1999). For lutein and zeaxanthin, they are abundantly constituted in the macula of human retina responsible for central vision and high visual acuity (Alves-Rodrigues and Shao, 2004; Yemelyanov, Katz and Bernstein, 2001). Yemelyanov, Katz, and Bernstein (2001) reported that the uptake and stabilization of these carotenoids is mediated by specific xanthophyll-binding proteins. With the purification and characterization of such binding protein using anion-exchange and gel filtration chromatography coupled with continuous photodiode-array monitoring for endogenously associated xanthophylls, they found two major bands at 25 and 55 kDa that co-eluted with lutein and zeaxanthin. Other potential mammalian xanthophyll-binding proteins such as albumin, tubulin, lactoglobulin, and serum lipoproteins possess only weak non-specific binding affinity for carotenoids. In addition, the visible absorption spectrum of those purified binding protein closely matches to that of the human macular pigment, and it is bathochromically shifted about 10 nm from the spectrum of lutein and zeaxanthin dissolved in organic solvents. These results are supported in afterward by the criticism on invertebrates and vertebrates carotenoid-binding proteins of Bhosale and Bernstein (2007).

There is a lack of literature data demonstrating to the identification and characterization of carotenoid-binding proteins in silk cocoons, unless the binding proteins from silkworm, *B. mori*. Tsuchida and others (2004) identified two carotenoid-binding proteins from the wild type of *B. mori* larvae. First, a lutein-binding protein, a 35 kDa and binds lutein in a 1:3 molar ratio, is synthesized in the midgut that equally distributed throughout the midgut and all developmental stages of larvae *B. mori* (Jouni and Wells, 1996). Another binding protein, designated as carotenoid-binding protein and binds lutein in a 1:1 molar ratio, was purified from the silk gland of *B. mori* (Tabunoki et al., 2004). It is interesting that this binding protein

is a novel number of the steroidogenic acute regulatory (StAR) protein family with the unique structural feature of a StAR-related lipid transfer domain and specific binding to carotenoids rather than cholesterol (Tabunoki et al., 2002). This protein plays a key role in the yellow cocoon pigmentation caused by carotenoids (Tabunoki et al., 2004; Tsuchida et al., 1998) with abundantly over 90% of lutein (Tsuchida et al., 1998). From these evidences, it can be proposed that the presence of lutein in the cocoon shells preferably exists in a protein-binding form.

2.3 Biological properties of lutein

2.3.1 Antioxidant activities

The antioxidant activities of carotenoids have been extensively described in previous reports (Krinsky, 1998; Stahl and Sies, 2003). Several distinct mechanisms are proposed by which carotenoids including lutein function as antioxidant. In generally, carotenoids are most likely involved in the scavenging of reactive oxygen species, singlet molecular oxygen ($^1O_2$), and peroxyl radicals. For $^1O_2$, it is capable of oxidizing nucleic acids, various amino acids, and unsaturated fatty acids, and usually formed through photochemical reaction by reacting with the singlet sensitizer molecule. In plants as well as human, carotenoids are the most effective quencher of $^1O_2$ that react via direct energy transfer reaction. Among the various carotenoids, xanthophylls as well as carotenes proved to be efficient quencher of $^1O_2$ (Baltschun et al., 1997; Conn, Schalch and Truscott, 1991). Due to the long conjugated double polyene nature of these molecules, they can return to ground state via vibrational and rotational interactions with the solvent systems or surrounding mediums. In addition to quenching $^1O_2$, they are able to interfere with radical-initiated reaction, particularly

with those that result in lipid oxidation (Krinsky, 1998; Woodall et al., 1997). The reaction of carotenoids with free radicals leads to electron transfer or possibly additional reaction. Moreover, there has been proposed the formation of a carotenoid-adduct radical or neutral carotenoid radical as well.

The antioxidant activities of lutein and its stereoisomers have been studied in various systems. Sujak et al. (1999) revealed that lutein and zeaxanthin were found to protect lipid membranes against free radical attack with almost the same efficacy. Similarly to the UV-induced lipid oxidation that slowed down to a very similar rate in the initial stage of 5-15 min of illumination, but zeaxanthin appeared to be better photoprotector during the prolonged UV exposure. The protein-binding form of zeaxanthin with glutathione *S*-transferase (GSTP1), purified from macula of the human eye, displayed synergistic antioxidant effects against 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH) and 2,2'-azobis(2,4-dimethylvaleronitrile) (AMVN). Moreover, non-dietary (3*R*,3'*S-meso*)-zeaxanthin was observed to be a better antioxidant than dietary (3*R*,3'*R-meso*)-zeaxanthin (Bhosale and Bernstein, 2005). Using the phtotochemiluminescence (PLC) assay and the β-carotene-linoleic acid model system (β-CLAMS), lutein showed a greater antioxidant activity than commom carotenoids, β-carotene and lycopene (Wang et al., 2006). In multilamellar liposomes, the antioxidant activity of seven carotenoids as inhibition of thiobarbituric acid reactive substances (TBARS) was ranked by Stahl's group (1998): lycopene > α-tocopherol > α-carotene > β-cryptoxanthin > zeaxanthin = β-carotene > lutein. They found the synergistic effect was most pronounced when lutein and lycopene were co-existed. In unilamellar liposomes, where lipid is more accessible to the aqueous medium, xanthophylls including lutein, zeaxanthin, and β-cryptoxanthin were

indicated to be very sensitive to degradation by radicals generated from iron and AAPH. Nevertheless, they were not protective against lipid peroxidation (Chen and Djuric, 2001). These finding indicated the pro-oxidant effect of those carotenoids when cooperated into the membrane structure. However, McNulty and co-workers (2007) proved that apolar carotenoids such as lycopene and β-carotene disordered the membrane bilayer and showed potent pro-oxidant effect (>85% increase in LOOH levels), while astaxanthin preserved membrane structure and exhibited significant antioxidant activity (40% decrease in LOOH levels). Evidences suggested that the protective effect as well as the effect on lipid peroxidation of carotenoids is related to the organization of the carotenoids in lipid membrane (Junghans, Sies and Stahl, 2001; McNulty et al., 2007: Stahl et al., 1998; Sujak et al., 1999; Sujak, Okulski and Gruszecki, 2000). For macular pigments, the difference in the protective efficacy of lutein and zeaxanhin was also found due to their different organization in lipid membrane structure. Zeaxanthin has been proposed in the protection of lipid phase against oxidative damage, and lutein in absorbing short wavelength radiation penetrating retina membranes (Sujak, Okulski and Gruszecki, 2000). There has been supported document by Junghans, Sies and Stahl in the next year, demonstrated that the filtering of blue light in lipid membrane was showed a greater efficacy by lutein compared to zeaxanthin, β-carotene, and lycopene, respectively. In retinal epithelial cells, lutein and zeaxanthin displayed the higher efficiency ratio of the solubilization compared to canthaxanthin and β-carotene in other membranes (Shafaa, Diehl and Socaciu, 2007). These incidents may attribute to the difference in the specific absorption and biological properties of a certain carotenoid. However, the physiological functions of individual carotenoids in biological systems have been

discussed. Those results were obtained *in vitro* study using a greater level of the free radicals as well as oxygen tensions than that found in biological system. To clearly understand the oxidative defense of those carotenoids in biological system, further study is, therefore, advantageous.

2.3.2 Antioxidant capacity assays

Due to the complexity of the compositions of foods as well as other plant and animal sources, it is difficult to separate each antioxidant compound and studying it individually. Therefore, the methods for determination of antioxidant capacity have been developed by many researchers for decades. Huang, Ou and Prior (2005) classified these assays into two types: assay based on hydrogen atom transfer reactions including inhibition of induced low-density lipoprotein auto-oxidation, oxygen radical absorbance capacity (ORAC), total radical trapping antioxidant parameter (TRAP), and crocin bleaching assays; and assay based on electron transfer including the total phenols assay by Folin-Ciocalteu reagent (FRC), Trolox equivalence antioxidant capacity (TEAC), ferric reducing antioxidant power (FRAP), "total antioxidant potential" assay using a Cu(II) complex as an oxidant, and DPPH·. Apart from those, other assay intended to measure the capacity of interest in which scavenging of biologically relevant oxidants such as $^1O_2$ and hydroxyl radical. In this study, we decided to use the stable DPPH· radical scavenging activity and FRAP assay, since these methods have been commonly used to evaluate antioxidant activity *in vitro* with several advantages as following; inexpensive, simply, high reproducible output, rapid, and accurate for measuring of antioxidant activity of plant and foods (Benzie and Strain, 1996; McInerney et al., 2007; Cheng, Moore and Yu, 2006; Sánchez-Moreno et al., 2006; Santas et al., 2008; Thaipong et al., 2006).

The FRAP assay is based on the reduction of a ferroin analog, the Fe^{3+} complex of tripyridyltriazine $Fe(TPTZ)^{3+}$, to the intensity blue colored Fe^{2+} complex of tripyridyltriazine $Fe(TPTZ)^{2+}$ by antioxidant in acidic medium with absorbance increase at 593 nm (Antolovich et al., 2001; Huang, Ou and Prior, 2005). Obtained results provide a very useful value of total antioxidant concentration without measurement and summation of the concentration of all antioxidants involved. The FRAP value can be expressed as μM Fe^{2+} equivalents or relative to an antioxidant standard.

DPPH· is one of a few stable and commercially available organic nitrogen radicals (Huang, Ou and Prior, 2005). It has an ultraviolet and visible absorption maximum at 515 (Huang, Ou and Prior, 2005) or 517 nm (Antolovich et al., 2001). However, to measure the value of the absorbance at 580 nm avoids the interference compounds with a chromophoric system of DPPH· as firstly described by Jiménez-Escrig et al. (2000). This assay performed in organic solvents that, consequently, may evaluate lipophilic antioxidants without any additional solubilizing agents. Evidences suggested that DPPH· scavenging capacity is highly dependent on the reaction time (Cheng, Moore and Yu, 2006; Sánchez-Moreno, Larrauri and Saura-Calixo, 1998). Thus, for a certain reaction, increase in the reaction time from 30 min to 40-1,400 min is required to reach the ready state of the reaction. The retention of DPPH· (%$DPPH\cdot_{rem}$) is proportional to the concentration of the antioxidants. The parameter EC_{50}, the concentration that causes a decrease in the initial DPPH· concentration by 50%, and T_{EC50}, the time need to reach the steady state with EC_{50} concentration, are obtained and highly dependent on the unit. Thus, the EC_{50} values are usually expressed in terms of moles of antioxidant per mol of DPPH· in the reaction medium.

The antiradical efficiency (AE) can be evaluated as new parameters to express the antioxidant capacity of a certain antioxidant, proposed by Sánchez-Moreno, Larrauri and Saura-Calixo (1998).

2.4 Factors affect lutein stability

In nature, lutein exists primarily in the more stable (all-*E*)-isomeric form. However, small amount of its (*Z*)-isomers also occur and significantly increase as a consequence of food preparation, processing, and storage. The highly unsaturated lutein is prone to isomerization and oxidation. Heat, light, acids, and adsorption on an active surface, such as alumina, promote isomerization of (all-*E*)-lutein to (*Z*)-isomers (Rodriguez-Amaya, 2001). Whereas, oxidative degradation is commonly caused and stimulated by available oxygen, light, enzymes, and metals as well as co-oxidation with lipid hydroperoxides (Dutta, Chaudhuri and Chakraborty, 2005; Rodriguez-Amaya, 2001). Formation of epoxides and apocarotenoids, carotenoids with shortened carbon skeleton, appears to be the initial step (Figure 2.4). They are somewhat sensitive to heat treatment, while lutein and hydrocarbon carotenoids such as α-carotene, β-carotene, ζ-carotene, lycopene, and phytoene survive the heat treatment (Khachik et al., 1992). Therefore, the total loss of color and biological activities are the final consequences once those oxidative products are fragmented to be a series of low molecular weight compounds.

The carotenoid composition of foods are affected by factors such as cultivar or variety, part of the plant consumed, stage of maturity, climate or geographic site of production, harvesting and postharvest handling, processing, and storage (Rodriguez-Amaya, 2001). Previous study, for instance, has been revealed the difference in the

carotenoid composition of a leafy vegetable (kale) as the effect of the cultivar differences, seasonal variations, and farming practices (Mercadante and Rodriguez-Amaya, 1991). In addition, by various means of cooking such as microwave heating, boiling, steaming, and stewing affect on the level of carotenoids in green vegetables and tomatoes as well (Khachik et al., 1992). Apart from food sources of lutein, considerable variations in lutein ester contents of marigold flowers have been established among 11 Chinese cultivars (Li et al., 2007). The study of carotenoid stability in the flowers of daylily (*Hemerocallis disticha*) has been also revealed the loss of carotenoids as the effect of various treatments, such as hot-air drying (50°C), freeze-drying, and soaking in a sodium sulfite solution (1% w/v) (Tai and Chen, 2000). As a result of various factors, affected on the stability of carotenoids including lutien, the extensive study of carotenoid degradation is therefore favorable in a certain system.

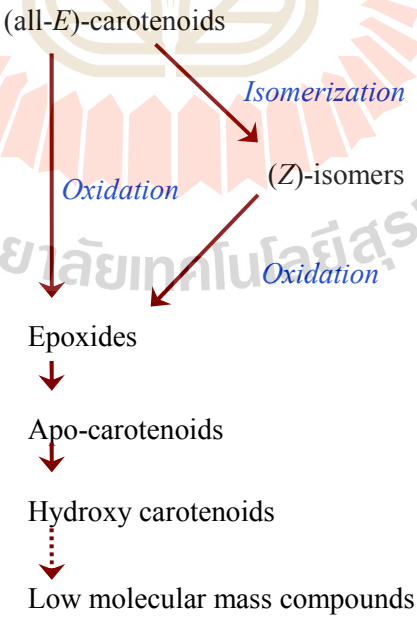

Figure 2.4 Possible scheme of carotenoid degradation.

From: Dutta, Chaudhuri and Chakraborty (2005)

2.5 Processing and storage of lutein in foods

2.5.1 Stability of lutein: Effect of thermal processing

Several studies have been demonstrated a consequence of food processing and storage condition in the considerable increment of (*Z*)-isomers of carotenoids including lutein (Aman, Schieber and Carle, 2005; Aman et al., 2005; Chen, Peng and Chen, 1996; Chen and Chen, 1993; Lee and Coates, 2003; Updike and Schwartz, 2003). Heat treatment is well known as one of the major effects contributing to degrade some isomeric carotenoids (Aman, Schieber and Carle, 2005). On the other hand, some of those carotenoids are lost during cooking, however in many cases they become more bioavailable after cooked (Deli et al., 2003; Tang and Chen, 2000). Nevertheless, the biological activity of different lutein stereoisomers presence in the functional foods is not well established. The assumption of the different existing forms of which may lead to their different bioavailability and physiological activity in per se. Besides, there has been the accumulating evidence of the role of lutein in prevention of AMD and the detection of (*Z*)-isomers of lutein in human (Khachik et al., 1995). Consequently, the interest in the impact of processing on the isomeric composition of lutein-rich natural products and lutein dietary supplements is increased (Deli et al., 2003; Schieber and Carle, 2005). The production of lutein-related products regularly involved thermal processing and storage that affects on lutein form and its stability. From these reasons, the study of lutein stereochemistry after being processed is therefore very important.

The recent study of Aman, Schieber and Carle (2005) evaluated the influence of thermal treatment on the degradation and isomerization of lutein appearing in spinach. They found the reduction of lutein by 16.7% after heating at 98°C for 60 min.

The loss of lutein by 28.0% and 7% was also found as the effect of food matrices like chloroplast isolates and lipids, respectively. In addition to thermal degradation, the *E*/Z isomerization of lutein was observed with the predominance of (9-*Z*) and (13-*Z*)-isomers which present in different proportion depending on the food matrices. The effect of thermal treatment on lutein stability in spinach was also revealed by Aman's group in the same year. More than 3% (w/w, dry basis) of natural presence of (9-*Z*)-isomers in fresh spinach substantially degraded after blanching with vapor at around 100°C for 2 min, in stead of the conversion of (all-*E*)-lutein to its *(Z*)-form. This predominant reduction of the (9-*Z*)-isomers was disagreed with the results from earlier group, unless insignificant change of the (13-*Z*)-isomers. This difference in results may attribute to the effect of food matrices, since lipids also took part in previous study.

Pasteurization is important to the stability of fruit and vegetable juices during transport and marketing. Nevertheless, decrease in the concentration of lutein and zeaxanthin during pasteurization has been reported. Gamma and Sylos (2007) revealed the effect of thermal pasteurization and concentration processes on the reduction of xanthophylls in Brazil Valencia orange juice. Thermal pasteurization was carried out at 95-105°C for 10 seconds and, for concentrated juice, held until the orange juice reached 66°Brix. After heat induction, they found the loss of lutein and zeaxanthin by 20% and 9%, and 17% and 24% in the pasteurized and concentrated orange juices, respectively. Among three main carotenoids found in the orange juice comprising lutein, *β*-cryptoxanthin, and zeaxanthin, the highest heat loss was observed for lutein and zeaxanthin. However, an inverse effect was found in Valencia orange juices, originated from USA (Lee and Coates, 2003) that contained violaxanthin and antheraxanthin as major carotenoid compositions. The loss of lutein and zeaxanthin

was lower than both major carotenoids after thermal pasteurization at 90°C for 30 seconds. The variation of that thermal degradation may attribute to the intrinsic stability of individual carotenoid. In case of violaxanthin, an epoxycarotenoid, it has been reported as one of the most labile carotenoids that easily isomerized in the presence of acid (Lee and Coates, 2003; Rodriguez-Amaya, 2001). These supported the proposed hypothesis that the degradation and isomerization of carotenoids including lutein and its isomers quit varies depending on their intrinsic structure and physical conditions such as their food matrix, composition of foods, and the interaction among existing carotenoids, or even between carotenoids and food matrix (Aman, Schieber and Carle, 2005; Dutta, Chaudhuri and Chakraborty, 2005; Rodriguez-Amaya, 2001; Schieber and Carle, 2005).

Thermal processing, affects on lutein and zeaxanthin isomerization in various vegetables, were also investigated by Updike and Schwartz (2003). The canning process at 121°C for 40 min enhanced the formation of (13-*Z*), (9-*Z*), and (9'-*Z*)-isomers of lutein that agreed with the study of Aman, Schieber and Carle (2005). Higher degree of temperature and longer treatment time may lead to extent the conversion of (all-*E*) to (*Z*)-form. Comparing to previous study where heating process at 98°C for 60 min was used, the severed heating as canning process caused the increase in the (13-*Z*) and both (9-*Z*), and (9'-*Z*)-isomers for 30 and 10 times, respectively. These evidences proved that the *E*/Z isomerization of lutein occurred during thermal processes, and (13-*Z*)-isomers were predominantly increased.

2.5.2 Stability of lutein: Effect of light exposure

In addition to thermal processing, illumination also facilitated the degradation and isomerization of lutein. Lutein solution and chloroplast isolate were used as the models for determination of illumination effect on the stability of lutein (Aman,

Schieber and Carle, 2005). After all samples were exposed to fluorescent light (7,550 Lux) for 0, 0.5, 1, 2, 5, 10, 20 and 60 min, the lutein level continuously decreased to 80.4% of the initial content after 60 min. The isomeric composition of lutein exhibited a time-dependent degradation following exposure to the light. Besides, (13-*Z*)-lutein was more susceptible to formation than (9-*Z*)-lutein (Aman, Schieber and Carle, 2005; Lin and Chen, 2005; Tang and chen, 1999). Remarkably, the illumination of chloroplasts was associated with a considerable increase in lutein level from the initial content for 9.6% and 0.3% during the first 10 min, and when reached 60 min, respectively (Aman, Schieber and Carle, 2005). These may explain by the nature existing of lutein in chloroplasts that lutein as well as other xanthophylls predominantly associated with light-harvesting proteins as pigment-protein complexes, acting as a concomitant pigment for light harvesting. Evidences suggest that lutein-binding protein and lutein-binding lipid complexes were less susceptible to light and heating than free lutein (Rodriguez-Amaya, 2001; Subagio, Wakaki and Morita, 1999). These have been the important factors promoting the stability of lutein against its physiological environment. Moreover, the parent carotenoids of lutein such as phytene, lycopene, α-carotene, and α-cryptoxanthin or zeinoxanthin may be altered to lutein by enzymatic reaction (Rodriguez-Amaya, 2001). When compared to free lutein, the greater retention of lutein in chloroplast isolates was therefore observed. However, comparing to the thermal impact on the lutein stability, the effect of illumination was insignificant.

2.5.3 Stability of lutein during storage

Freeze-drying is required for the production of lutein supplement and carotenoid-rich beverages such as carrot, tomato, and orange juices, since the conventional spray-drying diminish the stability of powders (Tang and Chen, 2000).

Although non-thermal process can minimize the loss of lutein, storage conditions such as storage temperature and illumination still have potentially effect on the stability of lutein. Like food processing, heating, and light exposure may be the major effects on the retention of lutein during storage (Rodriguez-Amaya, 2001). Tang and Chen extracted carotenoids from carrot pulp wastes with the mixture of acetone and hexane in the ratio of 18:27 (v/v) and then saponified with 40% metanolic potassium hydroxide for 2 hours at room temperature prior to freeze-drying under a vacuum 0.4 mm Hg for 24 hours, using sucrose and gelatin as the substrates. After storage under light and in the dark at 4, 25 and 45°C for 12 weeks, they found the greater destruction of (all-*E*)-lutein along with increase in the storage time. The (13-*Z*)-lutein was more readily formed than (9-*Z*)-lutein during storage in the dark, and they both showed the greater amount at higher storage temperature. This result corresponded to the study by Chen, Peng and Chen in 1996, where carrot juice was used and prepared by acidification and pasteurization before exposure to light and dark storage at 4, 25, and 35°C for 3 months. In addition, the recent study reported similar results, when using tomato juice which was processed by hot-breaking of tomatoes at 82°C, screening, heating at 121°C for 40 seconds and then storing in the dark and under light at 4, 25 and 35°C for 12 weeks (Lin and Chen, 2005). The lower formation of those (9-*Z*)-isomers probably attributed to their higher activation energy of isomerization comparing to the central (*Z*)-isomers, such as (13-*Z*)-lutein (Tang and Chen, 2000). In the juice, the presence of suspended particles and large molecules may offer protection for (9-*Z*)-lutein isomerization. Besides, the initial concentration of lutein in the juice was too low, and hence the relative concentration change of (9-*Z*)-lutein was small (Chen, Peng and Chen, 1996). However, the type of major isomers formed may be inconsistent, depending on storage conditions.

Table 2.3 Rate constants of lutein, α-carotene, and β-carotene in freeze-dried carotenoid powder during storage at various temperatures.

	Rate constant (day^{-1})		
Temperature	Lutein[a]	α-Carotene[b]	β-Carotene[c]
4ºC (dark)	0.004	0.013	0.015
25ºC (dark)	0.006	0.020	0.024
45ºC (dark)	0.009	0.032	0.037
25ºC (light)	0.013	0.039	0.043

Note: [a] Lutein includes (all-*E*)-lutein and its (*Z*)-isomers. [b] α-Carotene includes (all-*E*)-α-carotene and its (*Z*)-isomers. [c] β-Carotene includes (all-*E*)-β-carotene and its (*Z*)-isomers.

From: Gama and Sylos (2007)

Table 2.3 shows the degradation rate constants of the total amount of (all-*E*) plus (*Z*)-forms of lutein, comparing with α- and β-carotene, during storage. The degradation rate of each pigment was assessed to fit a first-order model. For lutein, the degradation rate was less than α- or β-carotene, which may be contributed to the formation of lutein-gelatin complex in the powder (Tang and Chen, 2000) and the stability of powder was, therefore, greatly enhanced. Moreover, with increasing of storage time and temperature, both the brightness and yellow color of powder decreased, similarly to the juice where the color became lighter (Lin and Chen, 2005; Tang and Chen, 2000). These may mainly cause by the degradation of carotenoids or the formation of (*Z*)-isomers (Rodriguez-Amaya, 2001; Tang and Chen, 2000). However, the loss of lutein bioavailability may consequently limit, since the degradation of lutein and its isomers during storage was insignificantly changed.

CHAPTER III

MATERIALS AND METHODS

3.1 Materials

Xanthophyll (approx. 90% of (all-*E*)-lutein; Fluka, Switzerland) and mixed isomer carotene (Sigma-Aldrich, St. Louis, USA) were used as standard compounds for identification and quantification. (all-*E*)-*β*-Apo-8'-carotenal (Sigma-Aldrich) was used to check retention times on the HPLC. Iron (II) sulphate heptahydrate, iron (III) chloride heptahydrate, 2,4,6-tris(2-pyridyl)-*s*-triazine (TPTZ), 2,2-diphenyl-1-picrylhydrazyl stable radical (DPPH˙), *α*-tocopherol, butylated hydroxytoluene (BHT), and butylated hydroxyanisole (BHA) were purchased from Sigma-Aldrich. A 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) was purchased from Fluka, USA. All carotenoids and other antioxidants were stored at -20ºC or below in nitrogen-packed vials, and solutions were made immediately before use. Sephacryl S-200, strong anion exchange QXL-1ml column, weak anion exchange DEAE Hi-Trap 1-ml column, and pre-stained molecular weight markers were obtained from Amersham Biosciences, UK. A standard protein, bovine serum albumin (BSA) was purchased from Acros Organics, USA. HPLC grade solvents acetronitrile, methanol (MeOH), ethyl acetate, and *n*-hexane (Carlo Erba Reageni, Italy) were used without further purification. All other chemicals and solvents were of analytical reagent grade, unless otherwise mentioned.

3.2 Yellow silk cocoons

The yellow silk cocoons of a polyvoltine race (called locally as Nangnoi) of Thai silkworms, *B. mori*, without pupa inside were purchased from silk producers in Nakorn Ratchasima province, the northeast of Thailand. Foreign matters and dirty cocoons were selected out. Clean and dry cocoons were cut into pieces in which average length was 8 mm and kept out of oxygen and light at room temperature.

3.3 Nomenclature

The term *all-E* (all-*trans*) and *Z* (*cis*) refer to the geometrical isomers of carotenoids. Throughout this manuscript, the common names of lutein and zeaxanthin have been used instead of their correct systematic names, and unless specified, these names refer to the combined *all-E* (all-*trans*) and *Z* (*cis*) isomers of these carotenoids. The chemical structures and the correct systematic names for the carotenoids discussed in the text are shown in Figure 3.1.

3.4 Pigment extraction

Yellow silk cocoons were firstly degummed to partially remove glutinous silk protein sericin before extracting of yellow pigments with various solvent systems under dark. The pieces of yellow silk cocoons were soaked into distilled water in a ratio of 1:30 prior to heat at 121ºC for 15 min. After heated, degummed cocoons and degumming solution, called pigmented-sericin solution, were separated off and then kept at 4ºC. Pigment extraction was done according to the method of Lin and Chen (2003) with modification. To determine the extraction efficiency, the degummed cocoons were extracted for pigments with seven solvent systems including (S1)

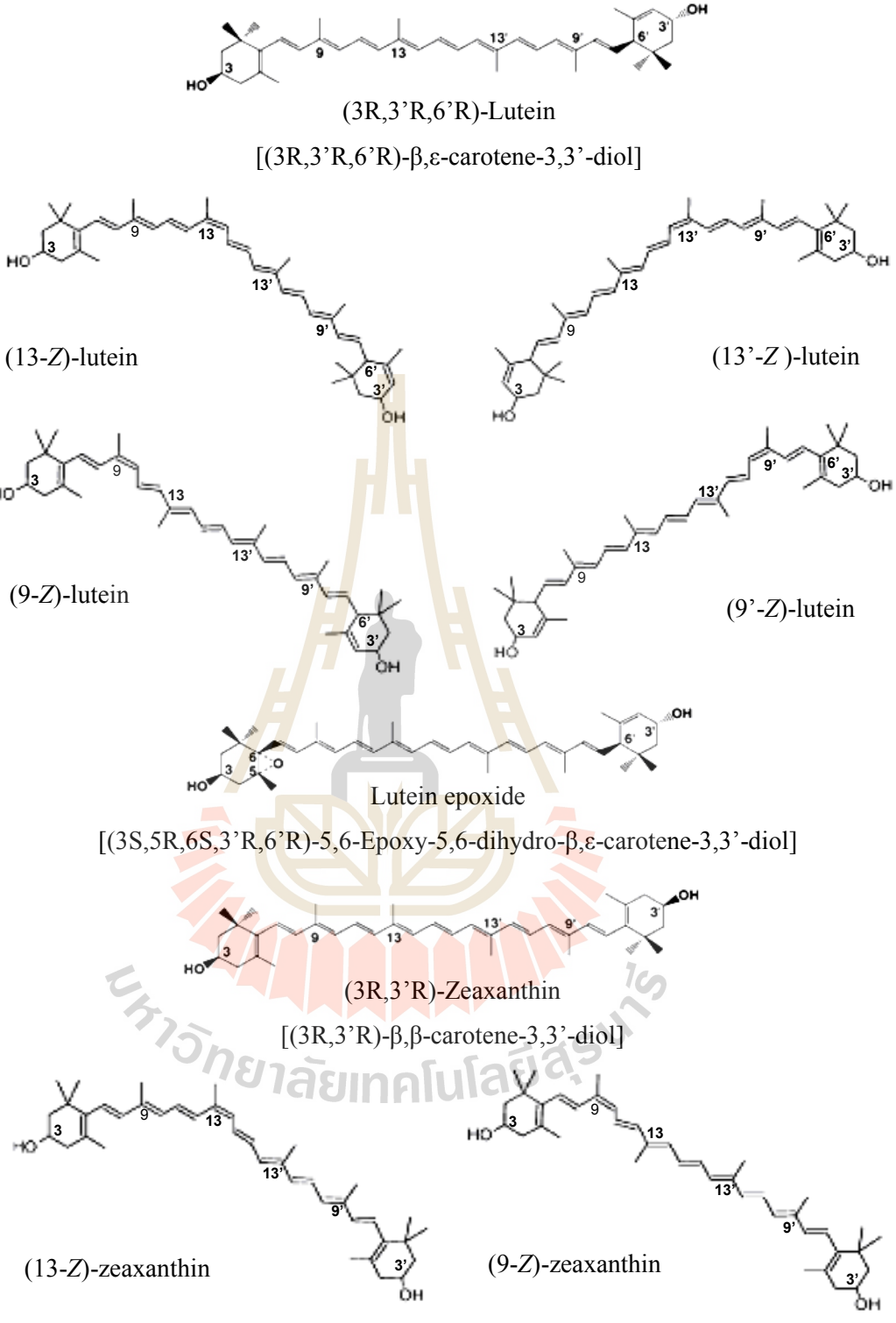

Figure 3.1 Chemical structures and systematic names of lutein, zeaxanthin, and their geometrical isomers and isomerized products.

From: Humphries and Khachik (2003); Updike and Schwartz (2003)

hexane/ethanol (3:4, v/v), (S2) hexane/acetone (5:3, v/v), (S3) hexane/acetone/ethanol (3:1:2, v/v/v), (S4) hexane/ethyl acetate (1:1, v/v), (S5) ethyl acetate (100%) (Aman et al., 2005; Hojnik, Škerget and Knez, 2008; Lin and Chen, 2003), (S6) hexane (100%) (Hojnik, Škerget and Knez, 2008; Jouni and Wells, 1996; Navarrete-Bolaňos et al., 2005), and (S7) hexane/ethanol/ethyl acetate (3:2:1, v/v/v).

The degummed cocoons (3 g of initial weight) were placed in a 250 mL Erlenmeyer flask and mixed with 90 mL extraction solvent as described above, containing 0.1% (w/v) of BHT and BHA each. The mixture was shaken in a shaker (KS 501, IKA, Germany) at 140 rpm/min for 2 hours under dim light at room temperature. An organic solution was collected and kept in an amber glass sample bottle at 4ºC. The degummed cocoons were repeatedly extracted three times under the same condition. The organic solutions were pooled and partitioned in an amber glass separating funnel by adding 100 mL of 10% (w/v) aqueous NaCl solution. For the extract solutions in acetone based extracting solvents (S2 and S3), a 100 mL of distilled water was added in order to remove acetone. The supernatant was collected and the aqueous phase was again extracted with the same extracting solvent (20 mL) until colorless. The pooled upper phases were dried with anhydrous sodium sulfate (2 g) and evaporated to dryness under vacuum at ≤35ºC. The residual was dissolved in hexane/ethyl acetate (3:1, v/v) and made up to a volume of 5-10 mL depending on the carotenoid content. An obtained crude extract was filtered through a 0.45 µm PTFE syringe filter and then kept under nitrogen gas in the dark at -20°C until analysis. The extraction efficiency of the extracting solvents was compared by means of spectrophotometrical quantification of the amount of total carotenoids and lutein content. The UV/VIS absorption spectra of the pigment extracts was determined as first criteria of identification and characterization of the carotenoid compositions.

3.5 Effect of degumming process on the efficiency of pigment extraction

Pigment extractions were carried out on both degummed cocoons and pigmented-sericin solutions, obtained from the degumming process with various heating conditions. A 3 g of yellow silk cocoons was soaked in 90 mL of distilled water and heated at 85, 105 and 121°C for 15, 30, 60, 90 and 120 min excepting for 15 min at 85°C due to poor degumming capability of such condition. Samples from each batch were filtered through sterile cheesecloth directly into 100-mL volumetric flasks and made up to volume with distilled water. In this process, pigmented-sericin solutions and degummed cocoons were obtained. To compare the influence of different degumming processes on the efficiency of pigment extraction, the degummed cocoons were fourfold extracted for the pigments as previous described by using hexane/ethanol/ethyl acetate (3:2:1, v/v/v) with 0.1% BHT (w/v). For the pigmented-sericin solution, pigment extraction was done according to methods modified from previous published data (Lakshminarayana et al., 2008; Prommuak, De Eknamkul and Shotipruk, 2008; Schäffer et al., 2008). A 40 g of the solution was extracted for the pigments in an amber glass separating funnel. A 60 mL of ethanol was added with vigorous shaking for 1 min to denature protein structure of sericin. The mixture was allowed to stand 10 min before extracting with 40 mL of hexane/ethyl acetate (3:1, v/v). The partition was done with 100 mL of 10% (w/v) aqueous NaCl solution and an upper phase was collected. An aqueous phase was repeatedly extracted for three times, or until colorless. The pooled organic phases were evaporated to dryness as the method mentioned above. The residue was prepared for the solution of the pigment extract in hexane/ethyl acetate (3:1, v/v). The total

amount of carotenoid and lutein in the extracts were quantified spectrophotometrically. The ratio of lutein to sericin content (L/S ratio) was determined to represent a proportion of lutein existing in the pigmented-sericin solutions.

3.6 Extraction of total carotenoids from yellow silk cocoons

In order to determine the total amount of carotenoids within yellow silk cocoons, the pigments were totally removed from the cocoons by repeated extractions. Sample (1±0.0002 g) of the cocoons was degummed with 30 mL of distilled water at 121°C for 15 min. The degummed cocoons (P1) were fourfold extracted with 30 mL of hexane/ ethanol/ethyl acetate (3:2:1, v/v/v) with 0.1% BHT (w/v) or until colorless. For the pigmented-sericin solution (P2), the extraction was done in an amber glass separating funnel by using 60 mL of the same extracting solvent. The partition was done separately and the upper phase was evaporated to dryness under vacuum as previous described. The residues were dissolved in HPLC grade *n*-hexane/ethyl acetate (3:1, v/v) and adjusted to final volume of 5 mL. After filtered through 0.45 µm filter membrane, the extract solutions were kept under nitrogen gas at -20°C until analysis. Carotenoids extracted from the degummed cocoons and the pigmented-sericin solutions were identified by HPLC. The absorption spectra and the concentration of carotenoids and lutein in the pigment extracts were determined in ethanol using spectrophotometer.

3.7 Determination of carotenoid content

Analysis of the extracts was performed using UV/VIS spectrophotometer (Libra S22, Biochrom Ltd., UK). Total carotenoids and lutein was calculated from multipoint calibration curve of standard lutein using absorbance value at 450 and 445 nm, respectively (Aman et al., 2005; Khachik, Bernstein and Garland, 1997; Corts et al., 2004; Kimura et al., 2007; Rodriguez-Amaya, 2001; Rodriguez-Amaya and Kimura, 2004). The light absorption characteristic of the pigment extracts was evaluated in ethanolic solution at 290-600 nm as the first criteria for identification and characterization of carotenoids (Zang, Sommerberg and Frederik, 1997). Lutein as well as other carotenoids was primary identified by the UV/VIS absorption spectra including the wavelengths of maximum absorption (λmax) and the spectral fine structure (Figure 3.2), expressed as %III/II in Eq (3.1):

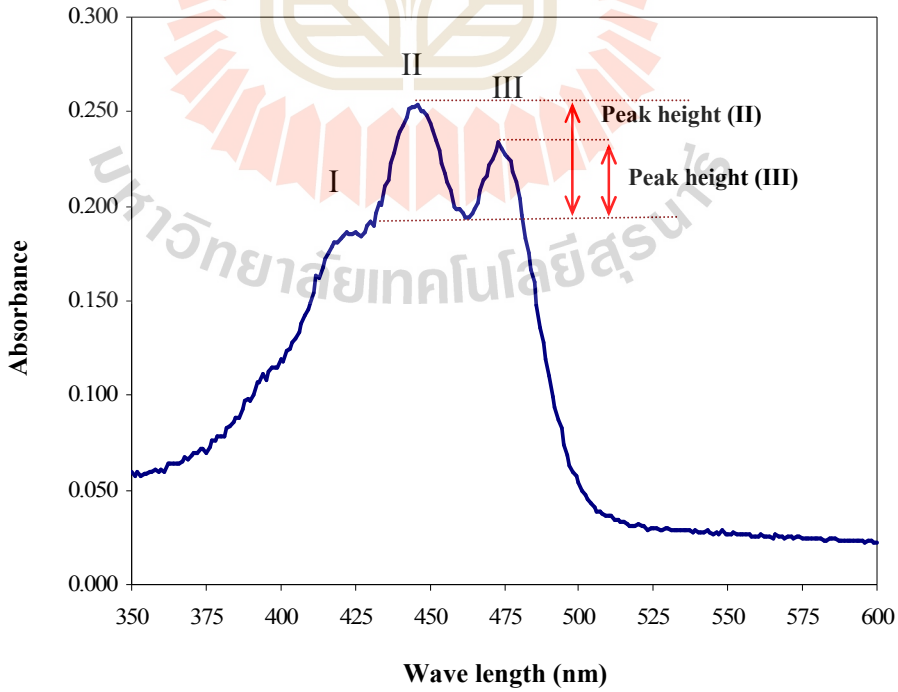

Figure 3.2 Calculation of %III/II as identification of spectral fine structure.

$$\%III/II = \frac{(A_{III} - A_B)}{(A_{II} - A_B)} \times 100 \quad (3.1)$$

where %III/II is the ratio of the height of the longest-wavelength absorption peak, designed as III, and that of the middle peak, designed as II, taking the minimum between the two peaks of baseline. Their UV/VIS absorption spectra were compared with authentic standards and literature data.

3.8 HPLC analysis of carotenoids

3.8.1 Preparation of standards

(all-*E*)-Lutein and (all-*E*)-carotenes were treated separately by iodine-catalyzed photoisomerization to obtain the mixtures of stereoisomers as the method described by Aman et al. (2005) with some modification. The stock solutions of standard lutein (50 μg mL^{-1}) and carotene standard (20 μg mL^{-1}) in HPLC grade hexane/ethyl acetate (2:1, v/v) were freshly prepared. A 500 μL of iodine in hexane (1 mmol L^{-1}) was added in each carotenoid solution to reach final iodine concentration about 1-2% (w/w) of the carotenoid. The solutions were exposed to fluorescent light (1,500 Lux) in a photostability chamber (Newtronic® NEC 103RSPSI, India) for 30 min at 20ºC. After illumination, the solutions were washed twice with sodium thiosulfate ($Na_2S_2O_3$) solution (1 mol L^{-1}) immediately. An organic phase was collected and made up to a volume of 5 mL. The residue was filtered through a 0.45 μm pore syringe filter into an amber vial for the analytical separation. Those standard carotenoid solutions without illumination were used for comparison.

3.8.2 Separation and identification of carotenoids in yellow silk cocoons

The qualitative and quantitative analysis of carotenoid composition in the

pigment extracts were performed using reversed-phase HPLC. The analyses were carried out on an Agilent HP 1100 series HPLC system, equipped with a quaternary solvent delivery system, a degasser, a thermostatic autosampler, a temperature-controlled column thermostat, a Photodiode-Array Detector GB57AA, and data acquisition system ChemStation software (Hewlett-Packard GmbH, Waldbronn, Germany). Analytical separations were performed under a LiChrospher® 100 reversed-phase C_{18} column (250 mm length x 4.6 mm internal diameter; 5 µm spherical particle) (Merck KGoA, Germany) at a column temperature of 20˚C. The column was protected with a C_{18} guard cartridge (30 mm length x 4.6 mm i.d.; 5 µm particle size). For simultaneous comparison, a reversed-phase C_{30} carotenoid column (250 mm length x 4.6 mm i.d.; 5 µm particle diameter) (YMC, Wilmington, MA, USA) was replaced for another chromatographic separation. The binary mobile phase consisted of solvent A (acetonitrile/methanol, 9:1, v/v) and solvent B (ethyl acetate) (Piccaglia, Marotti and Grandi, 1998; Tsao et al., 2004). Elution was carried out with a gradient program: 20% B in 15 min, 20% B to 50% B in 15-20 min, 50% B to 20% B in 20-25 min, and maintained at 20% B in 25-40 min. There was a 5-min post run under starting condition for re-equilibration. The flow rate was 0.5 mL min^{-1} for a total running time of 40 min. The injection volume was 10 µL, and eluting compounds were detected at 445 and 450 nm for monitoring lutein and other carotenoids, respectively. The photodiode array spectrum of the peaks of interest was scanned from 290 to 600 nm.

The carotenoids were identified using the HPLC system software by comparing their retention time to standard (all-*E*)-lutein, (all-*E*)-*α*-carotene, and (all-*E*)-*β*-carotene. Besides, their UV/VIS absorption spectra (λ_{max} and spectral fine

structure), obtained spectrophotometrically and by the photodiode-array detector, were compared to those of carotenoid standards and known published data. *β*-Apo-8'-carotenal was used as an internal standard to control the analytical process. The quantification was done by external standard calibration, based on peak areas. Duplicate analyzes were performed and the mean value was determined.

3.8.3 Separation of carotenoid stereoisomers

The separation of carotenoid stereoisomers was accomplished using a 5 µm particle diameter polymeric reversed-phase C_{30} carotenoid column (YMC; 250 mm length x 4.6 mm i.d.) (Aman et al., 2004; Aman et al., 2005; Young et al., 2007). Column temperature was maintained at 20˚C and a solvent elution was conditioned at a flow rate of 0.5 mL min^{-1}. Aliquots of 10 µL of the pigment extracts and standard solutions were separated within 80 min. A gradient program consisted of solvent A (acetonitrile/methanol, 9:1, v/v), solvent B (ethyl acetate), and solvent C (methanol). Elution was performed by using 26.6% C, maintained isocratically until the end of the run, with a gradient program: 10% B isocratically for 5 min, 10% B to 20% B in 5 min, 20% B isocratically for 25 min, 20% B to 50% B in 5 min, 50% B to 20% in 10 min, 20% B isocratically for 10 min, 20% B to 10% B in 1 min, and 10% B isocratically for 19 min. The detection was set at 445 and 450 nm and the photodiode array spectrum of the peaks of interest was scanned from 290 to 600 nm. Another chromatographic separation was done under a reversed-phase C_{18} column (250 mm length x 4.6 mm i.d.; 5 µm spherical particle) (Corts et al., 2004; Updike and Schwartz, 2003) with the same condition in order to compare the resolution.

Tentative identification of the (*Z*)-geometrical isomers of (all-*E*)-lutein and (all-*E*)-zeaxanthin was achieved by comparison of retention times, elution pattern, and

UV/VIS absorption spectra to isomerized lutein standard and literature data. Quantification of (*Z*)-isomers of lutein and other carotenoids was based on the standard curve of standard (all-*E*)-lutein, and expressed in lutein equivalent concentrations. The parameters of capacity ratio (*k'*) and selectivity (α) were determined according to the following equations:

$$k' = \frac{(t_R - t_0)}{t_0} \quad (3.2)$$

$$\alpha = \frac{k'_{\text{behind-peak}}}{k'_{\text{earlier-peak}}} \quad (3.3)$$

where t_R and t_0 are the retention times of the compound of interest and an unretained peak, respectively, and $k'_{\text{behind-peak}}$ and $k'_{\text{earlier-peak}}$ are the capacity ratio of two adjacent peaks of standard xanthophylls and (*Z*)-geometric isomers (Chen and Chen, 1994; McCall, 1975; Nells and De Leenheer, 1983). These obtained values were used in order to compare chromatographic resolution in terms of column selectivity and the efficiency of separation under the C_{18} and C_{30} columns.

3.9 Stability test

The degradation of the total amount of (all-*E*) plus (*Z*)-forms of lutein in the pigment extract from yellow silk cocoons was determined based on the method, modified from previous reports (Aman, Schieber and Carle, 2005; Tang and Chen, 2000). The influence of temperature, light, and solvents on the degradation of lutein was investigated. To prepare stock solution of lutein, degummed cocoons, heating at 121ºC for 15 min, were extracted for the pigment as previously described procedure.

The mixture of hexane, ethanol, and ethyl acetate (3:2:1, v/v/v) without antioxidant was used as an extracting solvent. An obtained stock solution of lutein, prepared in hexane/ethyl acetate (2:1, v/v) with the initial concentration of 15.52 mg mL^{-1} was applied for all experiments. Furthermore, to investigate the stability of lutein existing in a form of sericin-lutein complex, those obtained degumming solution was used for thermal stability test.

3.9.1 Stability test of lutein in dried lutein extract

The effect of temperature and light on the degradation of lutein in dried pigment extract was investigated. An aliquot of stock solution of the extract (500 μL) was pipetted to 4-mL vial and dried under nitrogen gas. To avoid pigment degradation in the presence of oxygen, samples were flushed with nitrogen and sealed tightly. Four groups of the experiment were divided to incubate separately in an incubator for 12 weeks at 4, 25 and 55°C under dark, and 25°C under fluorescence light with averaged intensity over sample of 3,100-3,161 Lux. All treatments were done in triplicate. The initial concentration of lutein was measured spectrophotometrically and, thereafter, monitored every 2 weeks until termination. Samples of the lutein extract were prepared in ethanolic solutions and then scanned for visible light absorption spectra from 350 to 600 nm. Decrease in absorbance and shifts in wavelength maxima were indicators of lutein degradation. The reduction of lutein was reported as percent of initial concentration at absorbance 445 nm.

3.9.2 Thermal stability test of lutein in organic solvents

Thermal stability of lutein extract in the solutions of various organic solvents was investigated by using the method, adapted from previous studies (Craft and Soares, 1992; Milanowska and Gruszecki, 2005; Subagio, Wakaki and Morita, 1999).

Each 500 µL of the extract was dried under nitrogen gas, and followed by dissolving in a 2 mL of six solvent systems comprising hexane/ethyl acetate (2:1, v/v), hexane/ diethyl ether (2:1, v/v), hexane/ethanol (2:1, v/v), hexane/ ethanol (3:1, v/v), hexane (100%), and ethanol (100%). All samples were sealed tightly under nitrogen gas and shaken vigorously before being heated in an incubator at storage temperature of 55ºC. The UV/VIS absorption spectra from 350 to 600 nm were monitored over 12-week period. Two samples were randomly removed from the incubator every 2 weeks, and the remaining concentration of lutein was measured in ethanolic solution by using spectrophotometer. The retention of lutein content in each storage period was calculated as previous described.

3.9.3 Thermal stability test of lutein in sericin-lutein complex

Thermal degradation of lutein existing in the pigmented-sericin solution was evaluated at 25, 55, 85 and 100ºC with minimal illumination. A 20 g of the pigmented-sericin solution was placed into a screw-cap test tube (20 mm x 125 mm). Four groups of the samples were divided and, thereafter, incubated separately in an incubator (25ºC) and each temperature-controlled water bath for 12 hours. From each treatment, three samples were randomly removed and cooled immediately in an ice bath after 1 hour incubation and additionally every 2 hours until finished. Samples of the pigmented-sericin solution were extracted for lutein once finished in each treatment of the incubation. Unheated degumming solution was used for determination of the initial content of lutein. Each sample was mixed with 30 mL of ethanol and shaken vigorously before extraction with 40 mL of hexane/ethyl acetate (3:1, v/v) containing 0.1% (w/v) BHT. The mixture was partitioned with 50 mL of 10% aqueous NaCl solution, and an upper phase was collected. Aqueous phase was

re-extracted with the same solvent until colorless. Pooled upper phase was removed solvents and then prepared in hexane/ethyl acetate (2:1, v/v) as described previously. The retention of lutein content was measured at 445 nm using spectrophotometric method.

3.9.4 Degradation kinetic analysis

A plot of natural-log lutein concentration versus storage time was use to best fit a kinetic model and to obtain the degradation rate constants, k (Eq 3.4) using the following formula:

$$k = -\ln\left[\frac{CA}{CA_0}\right] x \frac{1}{t} \quad (3.4)$$

where CA, CA_0 and t are the remaining concentration of lutein after stored under each storage condition, the initial concentration of lutein, and storage time, respectively. The linear correlation between the stability data was evaluated by the coefficient of determination (r^2). The half-life was calculated from rate constants of degradation kinetic at each storage temperature. As the temperature effect on the degradation rate of lutein, the activation energy (Ea) of thermal destruction was determined according to Arrhenius equation as following:

$$\ln k = \ln k_0 - \frac{Ea}{RT} \quad (3.5)$$

where k is the degradation rate constant of lutein in each storage temperature, k_0 is the frequency factor, R is the gas constant, and T is absolute temperature.

3.10 Determination of antioxidant activity

3.10.1 Sample preparation

The concentrated solution of lutein, extracted from yellow silk cocoons, was prepared without adding of any antioxidant. Pigment extraction was performed according to the method described earlier. Degummed cocoons, heating at 121ºC for 15 min, were used for pigment extraction by using a solvent mixture of hexane, ethanol, and ethyl acetate (3:2:1, v/v/v). The initial concentration of stock solution of lutein was checked daily via spectrophotometry. A series of working solutions of lutein as well as other antioxidants including α-tocopherol, trolox, and BHT were made in ethanolic solution of 2.5% THF. In this study, the assay of antioxidant activity was minimally performed by triplicate analyzes of five different concentrations of each antioxidant. To avoid the loss of antioxidant activity, the solutions were prepared freshly and kept at 4ºC under dark until used.

3.10.2 DPPH˙ radical-scavenging capacity

The antioxidant activity of the pigment extract and all standard antioxidants in term of the radical-scavenging ability was measured according to a previously described method of Jiménez-Escrig et al. (2000) with some modification. Briefly, different concentrations of the antioxidants in 2.5% THF ethanolic solution (100 µL) was placed in a spectrophotometer cuvette, and a 0.25 mM ethanolic solution of DPPH˙ (2.0 mL) was added. The initial concentration was 0.5 µM for DPPH˙ in all reaction mixtures. The same volume of a 2.5% THF ethanolic solution and deionized water was used instead of the antioxidants as a control sample and reference, respectively. The decrease in absorbance at 580 nm was measured continuously with data capturing at 15-second intervals within 30 min by using double-beams UV/VIS

spectrophotometer (GBC UV/VIS 916, GBC Scientific Equipments, USA). Ethanol was used to zero the spectrophotometer. The DPPH· solution was freshly prepared and stored at 4ºC under dark until used in order to minimize the loss of free radical activity.

The antioxidant activity was expressed in the percentage of DPPH· remaining, compared to the initial concentration of the stable radical without antioxidant. The level of DPPH· remaining for each reaction was calculated according to the following equation:

$$\%\ \text{DPPH}^{\cdot}\ \text{remaining} = \frac{(A_{\text{sample}} - A_{\text{blank}})}{(A_{\text{control}} - A_{\text{blank}})} \times 100 \qquad (3.6)$$

where A_{sample}, A_{blank} and A_{control} stand for the absorbance at 580 nm of sample, blank, and control reactions at 30 min, respectively.

To estimate the total DPPH· scavenging capability of the pigment extract as well as others antioxidant sample, the % DPPH· scavenging (Chang, Moore and Yu, 2006) was determined according to the following equation:

$$\%\ \text{DPPH}^{\cdot}\ \text{scavenging} = \left[1 - \frac{(A_{\text{sample}} - A_{\text{blank}})}{(A_{\text{control}} - A_{\text{blank}})}\right] \times 100 \qquad (3.7)$$

where A_{sample}, A_{blank} and A_{control} represent the absorbance of the certain concentration of the pigment extract or a selected antioxidant, blank, and control reactions at 580 nm and the reaction time of 30 min, respectively.

The concentration of the antioxidants that cause a decrease in the initial DPPH· concentration by 50%, defined as EC_{50}, and the time taken to reach the steady

state at EC_{50} (T_{EC50}) were interpolated from the regression model of dose-response curve. To express the antioxidant capacity of a certain antioxidant, the antiradical efficiency (AE) (Huang, Ou and Prior, 2005; Jiménez-Escrig et al., 2000; Sánchez-Moreno, Larrauri and Saura-Calixto, 1998) was also evaluated from Eq (3.8).

$$AE = \left[\frac{1}{EC_{50}}\right] \times T_{EC50} \quad (3.8)$$

3.10.3 Ferric-reducing antioxidant power (FRAP) assay

The Ferric-reducing antioxidant power (FRAP) assay was carried out according to the method of Benzie and Strain (1996) with some modifications. The stock solutions included 300 mmolL^{-1} acetate buffer, pH 3.6 (3.1g $C_2H_3NaO_2 \cdot 3H_2O$ and 16 mL $C_2H_4O_2$ L^{-1} of buffer solution), 10 mmol L^{-1} TPTZ (2,4,6-tripyridyl-*s*-triazine) in 40 mmol L^{-1} HCl, and 20 mmol L^{-1} $FeCl_3 \cdot 6H_2O$. The FRAP reagent, working solution was freshly prepared by mixing 50 mL acetate buffer, 5 mL TPTZ solution, 5 mL $FeCl_3$ solution, and 6 mL deionized water, and then warmed to 37°C prior to use. For FRAP assay, 60 µL of the pigment extract in 2.5% THF in ethanolic solution was pipetted onto the side of a quartz cuvette and 2 mL FRAP reagent was vigorously added. Absorbance readings of the colored product, ferrous tripyridyltriazine complex, were initially taken after 0.21 seconds once mixed and then every 15 seconds during 10 min of the monitoring. All reactions were done at 37°C using Double-beams UV/VIS spectrophotometer (Model GBC UV/VIS 916, GBC Scientific Instruments, Australia) with temperature controlling unit. Absorbance (A_{593nm}) at 4-min readings were selected for calculation of FRAP values since the reaction of lutein was completed and reached plateau stage in 4 min after sample-

reagent was mixed. Distilled water and 2.5% THF in ethanolic solution were replaced the lutein extract in the reaction of the control sample. The assay of other antioxidants including α-tocopherol and BHT was compared as a commercial source of natural and an artificial antioxidant. The reaction was performed in triplicate for each independent experiment. The standard curve of ferrous sulfate ($FeSO_4 \cdot 7H_2O$) was prepared in the concentration of 0.1, 0.2, 0.4, 0.6, 0.8 and 1.0 mM. The FRAP value was calculated from the regression equation and expressed in the unit of mM Fe^{2+} L^{-1}.

3.11 Characterization of pigmented-sericin complexes

3.11.1 Sample preparation

The pigmented-sericins complexes were isolated from yellow silk cocoons via degumming process at 121ºC for 15, 30, 60, 90 and 120 min. In each condition, yellow silk cocoons were degummed with deionized water in a ratio of 1:30 under dark. Once each heating period of time reached, the mixtures were cooled to room temperature and the degumming solutions were separated. Deionized water was used for adjusting to a 100-mL final volume of each pigmented-sericin solution. All treatments were done in triplicate. The total level of protein in the pigmented-sericin solutions was determined using bicinchoninic acid (BCA) protein assay kit (Sigma-Aldrich). The assay was carried out at 37ºC for 30 min with bovine serum albumin (BSA) as a standard (Smith et al., 1985).

Further purification and concentration was carried out by ammonium sulfate precipitation with minimum illumination. Sufficient solid ammonium sulfate was added slowly to each pigmented-sericin solution, with continuous stirring, to achieve 45% saturation (Jouni and Wells, 1996). The suspension was maintained in an ice

bath for 30 min before centrifugation at 10,000 x *g* at 4ºC for 30 min. The pellet was discarded and resuspended in a trace amount of 20 mM Tris-HCl buffer, pH 7.0, containing 150 mM NaCl. The concentrated solution of pigmented-sericin complexes was filtered through a 0.45 µm regenerated cellulose syringe filter before used.

3.11.2 Anion-exchanged chromatography

For the characterization of lutein in protein-binding form or sericin-lutein complex, anion exchange chromatography, modified from Jouni and Wells (1996) was implemented. The fractionation was performed under AKTA explorer system (ÄKTAexplorer, GE Healthcare, UK), comprised of sample pump P-960, air sensor, detector UV-900, fraction collector Frac-950, and the Unicorn™ control system. An aqueous pigmented-sericin solution, obtained from each degumming condition was filtered through a 0.45 µm syringe filter membrane. Protein sample was loaded on a weak anion exchange DEAE Hi-Trap 1-ml column, pre- equilibrated with 10 mM BisTris-HCl, pH 7.0. The fractions were eluted using a 1 M NaCl linear gradient in the same buffer, and 5 mL fractions collected. All fractions were monitored for peptides, protein and lutein at 254, 280 and 460 nm, respectively. In order to optimize the separation, a strong anion exchange QXL-1ml column was replaced for comparison.

3.11.3 Gel filtration

The concentrated samples of the pigmented-sericin solutions in 20 mM Tris-HCl buffer, pH 7.0, containing 150 mM NaCl was characterized based upon their different molecular size. The sample was loaded onto a Sephacryl S-200 column (1.6 cm x 80 cm), packed following the manufacturer's instructions and pre-equilibrated with 20 mM Tris-HCl buffer, pH 7.0, containing 150 mM NaCl. Elution was

performed with the same buffer at a flow rate of 0.5 mL min^{-1} under AKTA explorer system as described earlier. All fractions were monitored for peptides, protein and lutein at 254, 280 and 460 nm, respectively, and 0.5-mL fractions were collected. The elution profile of the samples was evaluated.

3.11.4 Sodium dodesyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Protein patterns of the pigmented-sericin solutions were determined using SDS-PAGE (Laemmil, 1970). Stacking and separating gels were made of 4 and 12.5% polyacrylamide, respectively. Samples were solubilized in a treatment buffer containing 10% β-mercaptoethanol, followed by heating in boiling water for 3 min and cooled. Protein sample (15 µg) was loaded onto each lane on polyacrylamide gel. Gels were run at a constant voltage setting of 120V. Gels were stained with 0.125% Coomassie Brilliant Blue R-250 and destained in a solution containing 25% methanol and 10% acetic acid.

3.12 Statistical analysis

Results were presented as mean values and standard errors of the means. An analysis of variance was performed to test significance at $p<0.05$. All statistic calculations were done using SAS for Windows version 6.08 (SAS Institute, Inc., Cary, NC).

CHAPTER IV

RESULTS AND DISCUSSION

4.1 Extraction of lutein from yellow silk cocoons

4.1.1 Effect of extracting solvents on the efficiency of pigment extraction

For extraction experiments, the polarity of the extracting solvents was varied from non- to high-polar species in order to evaluate the efficiency of the pigment extraction, and also to extend the extraction selectivity. After degumming at 121ºC for 15 min, yellow silk cocoons were then extracted with seven solvent systems to obtain pigment including lutein. There were significant different concentration of lutein amongst pigment extracts (p<0.05). Using non-polar extracting solvent as hexane resulted in the lowest yield of lutein (Table 4.1). Whereas, the highest amount of lutein extracted from 100 g of dried cocoons was at 26.93 mg (0.027%), using an organic solvent combination of hexane, ethanol, and ethyl acetate (S7), followed by hexane/acetone (S2) and hexane/acetone/ethanol (S3), respectively. Thus, an efficient extraction of lutein can be achieved using these hexane-based solvent systems. For these solvent systems, the solvent strength values (ε^o) were calculated for 0.216-0.395. Increase in the ε^o values (0.507 and 0.580) by using hexane/ethanol (S1) and ethyl acetate (S5) resulted in moderate extractability of lutein. Similarly to less-polar species as hexane/ethyl acetate (S4) with the ε^o value of 0.295 that small amount of lutein was extracted. However, by the use of hexane/acetone (S2) with lower the ε^o value (0.216), higher amount of extractable lutein was obtained because of the difference in extracting selectivity of lutein.

Table 4.1 Concentration and spectral fine structure of lutein in yellow silk cocoons after extraction with various solvent systems.

Sample	λmax (nm)			%III/II [c]	Lutein content [a]			
					wet basis		dry basis	
	I [b]	II	III		(mg/100g)	(% w/w)	(mg/100g)	(% w/w)
S1	(420)	443	469	15 [a]	20.338 [c] ± 1.516	0.0203 [c] ± 0.0015	22.528 [c] ± 1.527	0.0225 [c] ± 0.0017
S2	(420)	443	469	11 [d]	23.787 [ab] ± 2.592	0.0238 [ab] ± 0.0026	26.348 [ab] ± 2.611	0.0264 [ab] ± 0.0029
S3	(421)	442	469	13 [c]	22.751 [b] ± 1.792	0.0228 [b] ± 0.0018	25.200 [b] ± 1.805	0.0252 [b] ± 0.0020
S4	(420)	442	469	13 [bc]	12.253 [e] ± 0.899	0.0123 [e] ± 0.0009	13.572 [e] ± 0.905	0.0136 [e] ± 0.0010
S5	(421)	443	469	14 [ab]	15.176 [d] ± 0.939	0.0152 [d] ± 0.0009	16.810 [d] ± 0.946	0.0168 [d] ± 0.0010
S6	(419)	442	467	12 [c]	5.114 [f] ± 0.126	0.0051 [f] ± 0.0001	5.664 [f] ± 0.127	0.0057 [f] ± 0.0001
S7	(420)	443	469	13 [c]	24.316 [a] ± 0.625	0.0243 [a] ± 0.0006	26.934 [a] ± 0.630	0.0269 [a] ± 0.0007

Note: [a] Average of triplicate determinations ± standard deviation. [b] Values in parentheses represent shoulder. [c] %III/II represents the spectral fine structure, the shape of the spectrum. Yellow silk cocoons were degummed at 121ºC for 15 min and than extracted with (S1) hexane/ethanol (3:4, v/v), (S2) hexane/acetone (5:3, v/v), (S3) hexane/acetone/ethanol (3:1:2, v/v/v), (S4) hexane/ethyl acetate (1:1, v/v), (S5) ethyl acetate (100%), (S6) hexane (100%), and (S7) hexane/ethanol/ethyl acetate (3:2:1, v/v/v). Different letters on the same column indicate significant differences ($p \leq 0.05$).

An influence of different solvent polarity on the variation of extraction efficacy was considerably found in this study. Although, hexane has been widely used for extraction of lutein from various natural sources (Kimura et al., 2007; Nelis and De Leenheer, 1983; Piccaglia, Marotti and Grandi, 1998; Tsao et al., 2004), the lowest yield of lutein using hexane as an extracting solvent was found in this study. Considering that natural presence of lutein in yellow silk cocoons was possibly different from those previous sources. Like in flowers and some ripened fruits, one or two hydroxyl groups on the chemical structure of lutein were esterified with lipids

(Khachik and Beecher, 1988; Piccaglia, Marotti and Grandi, 1998; Subagio, Wakaki and Morita, 1999; Tsao et al., 2004; Young et al., 2007). The solubility of lutein was consequently enhanced in non-polar solvents. However, different results were found in some natural sources of lutein (Alves-Rodrigues and Shoa, 2004). For instance, in dark green leafy vegetables, lutein improved its solubility in polar-modified organic solvents as a result of its different existence as either free form, or protein-binding complexes. In contrast to esterified form, it was difficult to dissolve in hexane as corresponding to the results, found in this study. Therefore, it should be markedly proposed that lutein existed in yellow silk cocoons as non-esterified form.

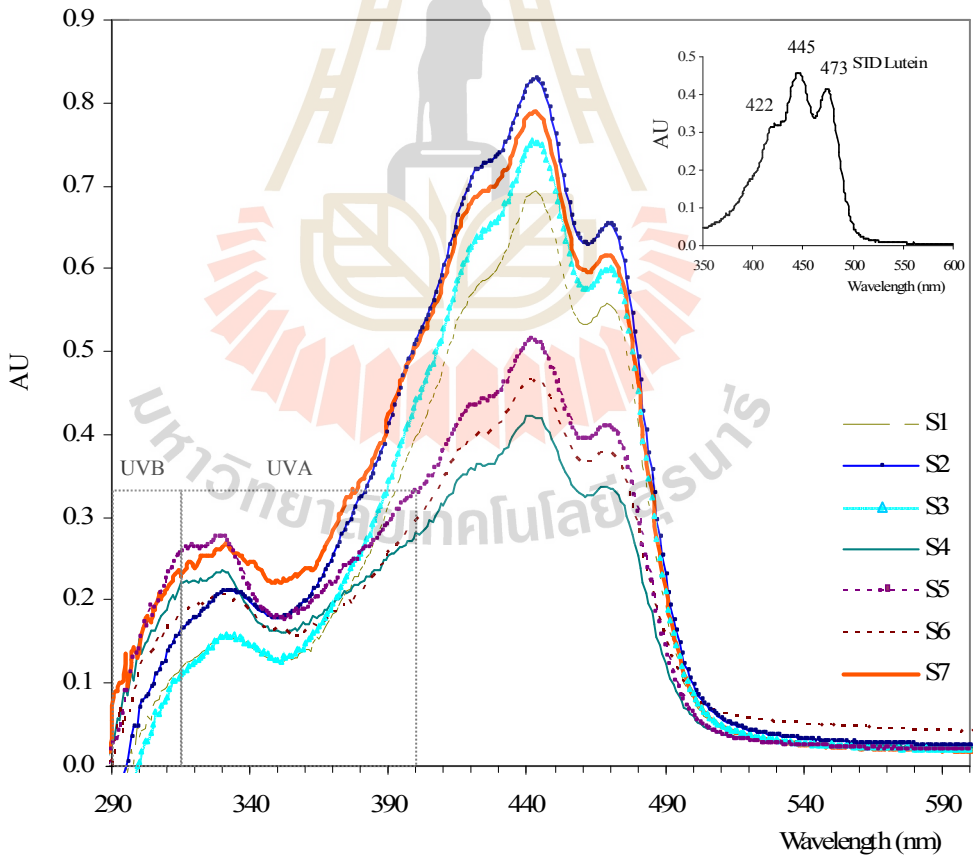

Figure 4.1 UV/VIS Spectra of pigment extracts from yellow silk cocoons with various extracting solvents.

As described in previous reports, the long conjugated double-bond system of carotenoids was known as their principal chromophore responding to absorb light strongly and exhibit intense main absorption bands in the visible region or, in a few case, UV region (Britton, Liaaen-Jensen and Pfander, 2004; Wrolstad et al., 2005). Consequently, the UV/VIS absorption spectrum was used in this study as one of the first criteria for identification and characterization of carotenoids (Britton, Liaaen-Jensen and Pfander, 2004; Rodrigures-Amaya, 2001; Wrolstad et al., 2005). Figure 4.1 shows the absorption spectra of the pigment extracting from degummed cocoons with various solvent systems. After scanning from 290 to 600 nm, the pigment absorbed maximally at three wavelengths of visible light at around 419-421, 442-443, and 467-469 nm (Table 4.1). Additional absorption peak was not observed when absorbance values were continuously monitored to reach 900 nm (data not shown). It was clearly that these three-maximum spectra of all pigment extracts were corresponding to the absorption characteristics of carotenoids (Rodrigures-Amaya, 2001). Moreover, even though the extraction selectivity was extended by using various extracting solvents, interestingly, similar profiles of the absorption spectra were found in all extracting conditions. These results indicated the identical composition of principle carotenoids that were found predominantly in yellow silk cocoons.

The absorption spectra present along with the λ_{max} values were expressed as the spectral fine structure (%III/II), the shape of the spectra that was considerable variation for difference carotenoids (Wrolstad et al., 2005). As previous studies, standard (all-*E*)-lutein and (all-*E*)-*β*-carotene give %III/II and λ-max values (the longest- to middle-wavelength) in ethanol of 60 and 26, and 445, 474 and 450, 477

nm, respectively (Aman, Schieber and Carle, 2005; Rodriguez-Amaya, 2001). Slightly different %III/II of standard (all-*E*)-lutein was found in this study. It was noticeable that the λ-max values of the lutein extracts were identical to that of (all-*E*)-lutein. However, the degree of %III/II was smaller than providing by (all-*E*)-lutein because of their impurity. As illustrated in Figure 4.1, additional absorption band appeared in the UV region around 310-340 nm representing to the existing of *trans*/*cis* (*E*/*Z*)-isomerization of lutein or other existing carotenoids (Rodrigures-Amaya, 2001). Therefore, difference in the %III/II values was related to the interference of existing (*Z*)-geometrical isomers that consequently altered the three-main absorption spectra of principal carotenoids.

4.1.2 Effect of degumming process on the efficiency of pigment extraction

The degumming process is generally used to eliminate sericin from silk fabric. In this study, the degumming process was performed in order to extend the pigment extraction from yellow silk cocoons. Regarding to this process, the cocoons were partially removed sericin via moist-heat treatment at 85, 105 and 121ºC for various time. Under high temperature (105-121ºC), traced amount of lutein removing with sericin resulted in yellowish degumming solutions, also called pigmented-sericin solutions. Degumming at 85ºC was not sufficient to remove any lutein, consequently, the degumming solutions obtained appeared in pale or gray color. There was almost totally lutein content remain in a part of degummed cocoons. Because of the degumming process, the efficiency of pigment extraction from silk cocoons was improved. The amount of extractable lutein was significantly different between degummed and raw silk cocoons ($p<0.05$). It was worth noting that extraction of the pigment from silk cocoons without degumming displayed a very low extracting

efficiency. Despite increase in the extraction time from 6 to 48 hours that only 0.0002% (w/w) of lutein was extracted from raw silk cocoons. In otherwise, more than 84-fold of extractable lutein were obtained when the degumming was conducted before extraction of the pigment. These results indicated the advantage of the degumming process that enhanced the extraction efficiency of lutein.

Table 4.2 shows the amount of sericin and lutein content in the degumming products, obtained from various heat treatments. As a result of mild heat treatment at 85ºC, sericin was slightly removed from silk cocoons. There was no significantly different among the total amount of sericin in the degumming solutions after heating for 30-120 min (p>0.05). However, the temperature rise from 85ºC to 121ºC contributed to enhance thermal hydrolysis of sericin resulting in the enlargement of sericin removal. Among various degumming conditions, the highest amount of sericin approximately 35.4-37.1% was obtained from both the conditions at 105ºC for 90-120 and 121ºC for 60-120 min. On account of degumming at 105ºC, the amount of sericin significantly increased along with increasing treatment time until reached 90 min (p<0.05). There was no significantly different sericin content between degumming conditions at 121ºC for 15-30 min and 105ºC for 60 min (p>0.05).

In addition to protein content, the amount of total solids in the degumming solutions was also determined. The results revealed that protein content was harmoniously related to the amount of total solids in the degumming solutions. Slightly different values between protein and total solid contents were observed at degumming temperature above 85ºC due to difference in analytical method (Smith et al., 1985; You et al., 2009; Walker, 2002). Therefore, sericin was implied to be a major component predominantly in the degumming solutions that agreed with

Table 4.2 Extraction efficiency of degumming process.

Temp. (ºC)	Time (min)	Degumming solutions [a]							Degummed cocoons [a]	
		Total solids (% w/w)		Protein content		Lutein content			Lutein content	
		degumming solution	cocoons	(g/100ml)	(% w/w, dry basis)	(mg/100g cocoons)	(% w/w, dry basis) cocoons	total solids	(mg/100g cocoons)	(% w/w, dry basis)
85	30	0.11 ^{j} ± 0.01	3.82 ^{j} ± 0.17	0.094 ^{e} ± 0.015	3.468 ^{e} ± 0.550	0.113 ^{i} ± 0.013	0.00011 ^{i} ± 0.00001	0.00053 ^{f} ± 0.00002	46.877 ^{a} ± 1.355	0.0516 ^{a} ± 0.0015
	60	0.13 ^{ij} ± 0.01	4.23 ^{ij} ± 0.29	0.133 ^{e} ± 0.023	4.923 ^{e} ± 0.861	0.041 ^{i} ± 0.017	0.00004 ^{i} ± 0.00002	0.00016 ^{f} ± 0.00002	45.269 ^{b} ± 1.004	0.0498 ^{b} ± 0.0011
	90	0.12 ^{j} ± 0.00	3.99 ^{j} ± 0.13	0.071 ^{e} ± 0.028	2.622 ^{e} ± 1.047	0.037 ^{i} ± 0.013	0.00004 ^{i} ± 0.00001	0.00017 ^{f} ± 0.00001	44.188 ^{c} ± 1.410	0.0486 ^{c} ± 0.0016
	120	0.13 ^{i} ± 0.01	4.28 ^{i} ± 0.26	0.118 ^{e} ± 0.017	4.367 ^{e} ± 0.612	0.037 ^{i} ± 0.011	0.00004 ^{i} ± 0.00001	0.00015 ^{f} ± 0.00001	41.859 ^{d} ± 1.188	0.0460 ^{d} ± 0.0013
105	15	0.41 ^{h} ± 0.02	13.80 ^{h} ± 0.68	0.418 ^{d} ± 0.070	15.442 ^{d} ± 2.590	9.110 ^{e} ± 0.737	0.00911 ^{e} ± 0.00074	0.00959 ^{c} ± 0.00266	29.297 ^{e} ± 2.450	0.0322 ^{e} ± 0.0027
	30	0.54 ^{g} ± 0.02	17.97 ^{g} ± 0.54	0.542 ^{c} ± 0.049	20.000 ^{c} ± 1.800	14.372 ^{d} ± 0.893	0.01437 ^{d} ± 0.00089	0.01467 ^{b} ± 0.00122	26.979 ^{f} ± 0.641	0.0297 ^{f} ± 0.0007
	60	0.55 ^{f} ± 0.01	18.48 ^{f} ± 0.48	0.808 ^{b} ± 0.142	29.851 ^{b} ± 5.249	18.593 ^{c} ± 1.201	0.01859 ^{c} ± 0.00120	0.01808 ^{a} ± 0.00122	20.198 ^{i} ± 0.372	0.0222 ^{i} ± 0.0004
	90	0.64 ^{e} ± 0.02	21.30 ^{e} ± 0.61	1.004 ^{a} ± 0.084	37.054 ^{a} ± 3.100	21.328 ^{a} ± 0.548	0.02133 ^{a} ± 0.00055	0.01812 ^{a} ± 0.00051	18.551 ^{j} ± 0.739	0.0204 ^{j} ± 0.0008
	120	0.69 ^{d} ± 0.02	22.85 ^{d} ± 0.58	0.996 ^{a} ± 0.062	36.786 ^{a} ± 2.272	19.748 ^{b} ± 0.571	0.01975 ^{b} ± 0.00057	0.01572 ^{b} ± 0.00051	18.649 ^{j} ± 0.887	0.0205 ^{j} ± 0.0010
121	15	0.71 ^{c} ± 0.01	23.58 ^{c} ± 0.44	0.803 ^{b} ± 0.075	29.651 ^{b} ± 2.780	9.652 ^{e} ± 0.360	0.00965 ^{e} ± 0.00036	0.00737 ^{d} ± 0.00028	25.633 ^{g} ± 0.550	0.0282 ^{g} ± 0.0006
	30	0.74 ^{b} ± 0.02	24.51 ^{b} ± 0.51	0.837 ^{b} ± 0.099	30.891 ^{b} ± 3.656	9.471 ^{e} ± 0.729	0.00947 ^{e} ± 0.00073	0.00706 ^{d} ± 0.00012	25.396 ^{g} ± 0.825	0.0279 ^{g} ± 0.0009
	60	0.75 ^{a} ± 0.01	24.90 ^{a} ± 0.39	0.997 ^{a} ± 0.083	36.800 ^{a} ± 3.077	8.466 ^{f} ± 0.216	0.00847 ^{f} ± 0.00022	0.00616 ^{d} ± 0.00014	25.021 ^{g} ± 0.559	0.0275 ^{g} ± 0.0006
	90	0.75 ^{a} ± 0.02	25.11 ^{a} ± 0.83	0.979 ^{a} ± 0.079	36.145 ^{a} ± 2.935	5.650 ^{g} ± 1.421	0.00565 ^{g} ± 0.00142	0.00409 ^{e} ± 0.00117	23.457 ^{h} ± 1.066	0.0258 ^{h} ± 0.0012
	120	0.76 ^{a} ± 0.02	25.17 ^{a} ± 0.78	0.959 ^{a} ± 0.106	35.405 ^{a} ± 3.919	4.878 ^{h} ± 0.612	0.00488 ^{h} ± 0.00061	0.00353 ^{e} ± 0.00050	22.833 ^{h} ± 0.296	0.0251 ^{h} ± 0.0003
RT [b]	6 [c]	- -	- -	- -	- -	- -	- -	- -	0.220 ^{k} ± 0.026	0.00022 ^{k} ± 0.00003
	6 [c]	- -	- -	- -	- -	- -	- -	- -	0.220 ^{k} ± 0.041	0.00022 ^{k} ± 0.00004
	48 [c]	- -	- -	- -	- -	- -	- -	- -	0.227 ^{k} ± 0.030	0.00023 ^{k} ± 0.00003

[a] Average of triplicate determinations ± standard deviation. [b] Extraction of lutein from raw silk cocoons at room temperature. [c] Extraction time (hours). Different letters on the same column indicate significant differences ($p \leq 0.05$).

previous studies (Freddi, Mossotti and Innocenti, 2003; Lamoolphak, De Eknamkul and Shotipruk, 2008; Vaithanomsat and Kitpreechavanich, 2008; Wu, Wang and Xu, 2007; Wu, Wang and Xu, 2008).

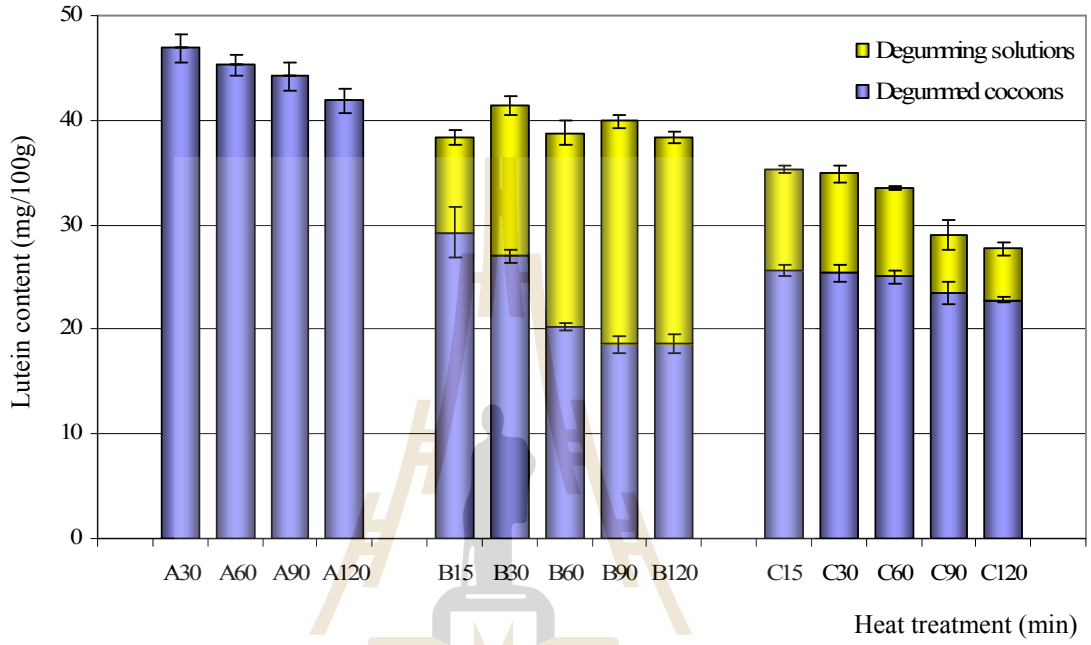

Figure 4.2 Effect of degumming processes on the efficiency of pigment extraction. symbols: A, B, and C represent degumming temperature at 85, 105 and 121ºC, respectively.

The concentration of lutein dissolving in the degumming solutions was investigated, in order to compare the degumming loss of lutein. As a result of degumming at 105ºC, the amount of lutein removed reached 0.009-0.021% (w/w) from the cocoons after treated for 15-90 min, and then tended to decrease as the treatment time increased. However, different results were observed when degumming was at 121ºC. From this condition, lutein content continuously decreased along with extending of heat exposure. As illustrated in Figure 4.2, most of lutein content still

remained in the cocoons, whereas increase in the loss of lutein dissolving into the degumming solutions was observed in degumming process at 105ºC. It was noticeable that lutein dissolved into the pigmented-sericin solutions with different proportions depending on degumming conditions changed. However, these solutions were not obtained from the degumming process at 85ºC due to poor efficiency of sericin removal of such condition. Therefore, to utilize the pigmented-sericin solution from yellow silk cocoons, the degumming at either 105 or 121ºC was potentially used depending on the pigmented ratio and expected degree of hydrolysis of sericin.

For degummed cocoon, a major part of lutein source, approximately 52.1-99.9% of lutein was remaining in the cocoons after degummed at 85-121ºC. Regarding to previous results, the improvement of pigment extraction from raw silk cocoons could be achieved by using the degumming process as pre-treatment. Because of degumming at 121ºC, degummed cocoons were likely to be softened. From this part, lutein was readily extracted with hexane/ethanol/ethyl acetate (3:2:1, v/v/v). Whereas, degummed cocoons obtaining from other conditions showed more hardened texture that, consequently, more repeated extraction of lutein was needed. These results probably attributed to the retention of sericin on the cocoon shells. Lee and Coates (2003) suggested that the molecular environment of sericin was affected by the addition of organic solvent as alcohol. Thus, the extraction of lutein from silk cocoons, degummed at 85-105ºC, was probably obstructed as affected by the packing of molecular chain of sericin remained.

The retention of lutein in the degummed cocoons, total lutein content, and the lutein to sericin (L/S) ratio were summarized in Table 4.3. The amount of extractable lutein was combined between which from pigmented-sericin solution and degummed cocoons. Obtained values of total lutein content showed significant difference among

the degumming conditions (p<0.05). To use the heat treatment at 85ºC was likely to be the best choice for increase in the yield of lutein extracting from the cocoons. In contrast with the pigmented-sericin solution that severer heat treatment was used for instead. Nevertheless, in this study, degumming at 85ºC was not used as pre-treatment process. Since, the extraction of lutein from degummed cocoons obtaining from this condition was time and solvent consumed. The L/S ratio was used in order to represent a proportion of lutein existing in the pigmented-sericin solutions. Therefore, in order to utilize lutein in a form of sericin-lutein complex, this value should be considered.

In addition to the proportion of lutein removing into the degumming solutions, the effect of heat treatment on the destruction of lutein was also observed in both degummed cocoons and pigmented-sericin solutions. The level of extractable lutein (Figure 4.1) indicated that the extension of the treatment time probably induced thermal degradation of lutein. Even though thermal treatment was realized to be essential for an efficient extraction of lutein, however, thermal degradation of lutein during degumming process was finally concerned. In this study, increase in the treatment time over 60 min at either 105 or 121ºC resulted in significant difference of lutein content in the degummed cocoons (p<0.05). However, there was no significantly different level of lutein remaining among the cocoons, degummed at 121ºC for 15-60 min (p>0.05).

To investigate the effect of light exposure, the extraction of lutein was carried out in raw silk cocoons without degumming. Astonishingly, exposure to indoor room light during extraction period for 8 hours did not affect on lutein content in the extracts. However, the extraction of lutein performing in this study was extensively

controlled under dark, since light is a factor stimulating the isomerization of lutein (Alves-Rodrigures and Shao, 2004; Astorg, 1997; Junghans, Sies and Stahl, 2001; Krinsky and Johnson, 2005; Molnár, 2009; Semba and Dagnelie, 2003; Stahl and Sies, 2003; Stahl and Sies, 2005).

Table 4.3 An influence of thermal treatment on lutein content in the degummed cocoons, lutein to sericin (L/S) ratio in the degumming solutions, and total lutein contents.

Temp.	Time	Total lutein content [a]		Retention of lutein [c]			L/S Ratio [b] (mg/kg)	
(ºC)	(min)	(mg/ 100g)	(% w/w, dry basis)	($\%_{tc}$)	($\%_{Tc}$)	($\%_{Total}$)	TP	TS
85	30	46.990 ^{a} ± 1.354	0.052 ^{a} ± 0.001	100.00	100.00	100.00	0.33	0.30
	60	45.310 ^{b} ± 1.014	0.050 ^{b} ± 0.001	96.57	96.57	96.42	0.08	0.10
	90	44.225 ^{b} ± 1.414	0.049 ^{b} ± 0.002	94.26	94.26	94.12	0.14	0.09
	120	41.896 ^{c} ± 1.192	0.046 ^{c} ± 0.001	89.30	89.30	89.16	0.08	0.09
105	15	38.407 ^{e} ± 2.738	0.041 ^{e} ± 0.003	100.00	62.50	81.73	5.90	6.60
	30	41.351 ^{c} ± 0.649	0.044 ^{d} ± 0.001	92.09	57.55	88.00	7.19	8.00
	60	38.791 ^{de} ± 1.165	0.041 ^{e} ± 0.001	68.94	43.09	82.55	6.23	10.06
	90	39.880 ^{d} ± 0.745	0.042 ^{e} ± 0.001	63.32	39.57	84.87	5.76	10.01
	120	38.397 ^{e} ± 0.584	0.040 ^{e} ± 0.001	63.66	39.78	81.71	5.37	8.64
121	15	35.285 ^{f} ± 0.824	0.038 ^{f} ± 0.001	100.00	54.68	75.09	3.26	4.09
	30	34.867 ^{f} ± 0.943	0.037 ^{fg} ± 0.001	99.07	54.18	74.20	3.07	3.86
	60	33.487 ^{g} ± 0.668	0.036 ^{g} ± 0.001	97.61	53.38	71.26	2.30	3.40
	90	29.106 ^{h} ± 1.003	0.031 ^{h} ± 0.001	91.51	50.04	61.94	1.56	2.25
	120	27.711 ^{i} ± 0.784	0.030 ^{i} ± 0.001	89.08	48.71	58.97	1.38	1.94

Note: [a] Total lutein content including in the degumming solution and the degummed cocoons were averaged from triplicate determinations ± standard deviation, in dry basis. [b] Values were calculated from the ration of lutein to total protein contents (TP) and total solids (TS) in 100 mL of the degumming solutions. [c] The amount of lutein extracting from the degummed cocoons represent in %remaining, compared to that of the shortest heating time of each temperature ($\%_{tc}$), and the lowest temperature and time ($\%_{Tc}$); total lutein content in yellow silk cocoons ($\%_{Total}$) represents in %remaining comparing to that of the lowest temperature and time. Different letters on the same column indicate significant differences ($p \leq 0.05$).

4.2 Quantitative analysis of lutein content in yellow silk cocoons

To determine the total lutein content, yellow silk cocoons were degummed at 85ºC for 30 min before complete extraction of lutein using hexane/ethanol/ethyl acetate (3:2:1, v/v/v). It was found that the extract contained lutein approximately 0.052% (w/w) in a dry basis (Table 4.4). Trace amount of lutein for 0.0001% distributed in a part of sericin solution, whereas remained lutein was found in the degummed cocoons. The absorption spectra of the extract were found the identical λ-max with smaller %III/II values than standard (all-*E*)-lutein. The results indicated that lutein be firstly identified as predominant carotenoids found in the extract. This might be the first report where the combination of lutein in yellow silk cocoons was explored. Unfortunately, comparison of the results obtaining from this source of lutein with literature values was, therefore, omitted.

Table 4.4 The amount of lutein found in yellow silk (*Bombyx mori*) cocoons and its spectral characteristics [a].

Sample [b]	λmax (nm)		%III/II	Lutein content			
	peak II	peak III		(mg/100g)		(%)	
				wet wt.	dry wt.	wet wt.	dry wt.
P1	445	473	41	46.559 ± 0.420	51.572 ± 1.338	0.04656 ± 0.00042	0.05157 ± 0.00047
P2	446	470	1	0.051 ± 0.006	0.057 ± 0.006	0.00005 ± 0.00001	0.00006 ± 0.00001
Total [c]				46.610 ± 0.426	51.628 ± 1.345	0.04661 ± 0.00043	0.05163 ± 0.00047

Note: [a] Average of triplicate determinations ± standard deviation. [b] Yellow cocoons in both parts of degummed cocoons (P1) and degumming solution (P2). [c] Total lutein content in yellow cocoons.

Table 4.5 Composition of lutein in different sources.

Source	Distribution of major carotenoids (mole%) [a]							Lutein composition [d]		
	Lutein/ Zeaxanthine	Lutein	Cryptoxanthins	Lycopenes	α-Carotene	β-Carotene	Neoxanthins/ violaxanthins	mg/100g fresh wt.	mg/100g dry wt.	References
Animal products:										
Yellow silk cocoons (*Bombyx mori*)	97	84	-	-	0	0	-	46.61	51.628	-
Egg york	89	54	4	0	0	0	8	3.766[(11)]-0.723	-	(9), (11)
Fruits/vegetables:										
Kale, raw	-	-	-	-	-	-	-	39.550[(1)]-12.310	-	(1), (3), (10)
"Winterbor" kale	-	-	-	-	-	-	-	15.10-6.90	71-112	(2)
Squash, pepper peel	-	-	-	-	-	-	-	30.180	-	(3)
Squash, pepper fresh	-	-	-	-	-	-	-	0.870	-	(3)
Spinach	47	47	19	4	0	16	14	11.940[(1)]-3.740	133.7[(4)]-34.9	(1), (3-6)
Leaf lettuce	15	15	36	0	16	0	33	2.635	-	(1)
Broccoli	22	22	49	0	0	27	3	2.445[(1)]-1.553	-	(1), (10)
Summer squash	52	47	24	0	0	5	19	2.125	-	(1)
Brussel sprouts	29	27	39	0	0	11	20	1.590	-	(1)
Peas (green)	41	41	21	0	0	5	33	1.350	-	(1)
Corn	86	60	5	0	0	0	9	0.884[(1)]-0.199	2.07[(6)]	(1), (5-6)
Green beans	0	0	28	0	0	0	72	0.640[(1)]-0.453	-	(1), (10)
Potato, fresh	-	-	-	-	-	-	-	0.410-0.250	-	(3)
Carrots, baby	-	-	-	-	-	-	-	0.358	-	(1)
Carrot	2	2	0	0	43	55	0	0.298	-	(5)
Cabbage	-	-	-	-	-	-	-	0.310	-	(1)
Oranges	22	7	12	11	8	11	36	0.187	-	(1)
Papaya	-	-	-	-	-	-	-	0.075[(1)]-0.044	-	(1), (10)
Peaches	13	5	8	0	10	50	20	0.04	-	(10)
Winter squash	-	-	-	-	-	-	-	0.038	-	(1)
Mango	18	2	4	6	0	20	52	0.020	-	(10)
Grapefruit, red	-	-	-	-	-	-	-	0.013	-	(1)
Tomatoes	6	6	0	82	0	12	0	0.130[(1)]-0.050	-	(1), (5)
Flowers:										
Marigold petals [b]	-	-	-	-	-	-	-	161-611	-	(7)
Marigold petals [c]	-	-	-	-	-	-	-	16.77-569.90	-	(8)
Marigold calyces [c]	-	-	-	-	-	-	-	0.38-18.60	-	(8)

Note: [a] The data from Sommerburg et al. (1998). [b] Lutein diesters in Chinese marigold flowers (*T. erecta*). [c] Lutein and lutein esters from marigold flowers, *Tagetes patula* and *T. erecta*. [d] Literature values refer to: (1), U.S. Department of Agriculture (1998), cited in Alves-Rodrigues and Shao (2004); (2), Lefsrud et al. (2007); (3), Tsao and Yang (2006); (4), Liu, Perera and Suresh (2007); (5), Konings and Roomans (1997); (6), Aman et al. (2005); (7), Li et al. (2007); (8), Piccaglia, Marotti and Grandi (1998); (9), Handelman et al. (1999); (10), Humphries and Khachik (2003); and (11), Schlatterer and Breithaupt (2006).

Table 4.5 shows the composition of lutein in yellow silk cocoons comparing with other common sources. It is interesting that the amount of lutein in yellow silk cocoons was found in the second highest, but absent of chlorophylls and lower impurities than that found in kale, the highest food source of lutein (Alves-Rodrigures and Shao, 2004; Deli et al., 2003). The chromatographic separation of the pigment extract proved that about 83.8% of lutein was found as a major pigment in yellow silk cocoons. In addition to lutein, about 13.3% of zeaxanthin and 3% of unidentified compounds were also present. Comparing to other sources, the highest distribution of lutein was found in yellow silk cocoons. As reported in previous studies, for instance, lutein content in corn and fortified egg yolk was 60% and 54%. Furthermore, zeaxanthin found in both was as followed 26%, and 34% respectively. Lower proportion of xanthophylls was found in fruits and vegetable due to their various carotenoid compositions. As indicated by these data, yellow silk cocoons were potentially used as a new rich source of lutein.

Excepting from food sources, marigold flowers (*Tagetes erecta*) are known as a rich source of xanthophylls ranging from 0.6 to 2.5% (w/w) on a dry basis (Tsao et al., 2004). This was around 10-20 times higher than that founded in food sources of lutein. From this reason, purified crystalline lutein, prepared from the pigment extract of marigold flowers, has been used in commercial dietary supplements for a recent decade. However, as a result of natural presence in lutein esters with high impurities (Piccaglia, Marotti and Grandi, 1998; Tsao et al., 2004; Xin-yu, Long-sheng and Chun-shan, 2005), extraction and purification of lutein from this source were complicated and costly. In marigold petals, lutein was found almost completely in diesters such as dimyristate, myristate-palmitate, dipalmitate, and palmitate-stearate,

majoring dimyristate and dipalmitate (83-92% of total esters) (Piccaglia, Marotti and Grandi, 1998; Tsao et al., 2004; Xin-yu, Long-sheng and Chun-shan, 2005), whereas in green vegetables, orange fruits, and egg yolk as well as yellow silk cocoons, it was existing as either non-esterified or protein-binding form (Hojnik, Škerget and Knez, 2008; Jouni and Wells, 1996; Tabunoki et al., 2002; Tabunoki et al., 2004). Thus, saponfication with alkaline solution was generally performed to enhance the pigmentation value of the extract from marigold petals (Hadden et al., 1999). Additionally, crystallization process was also need for removal of impurities before used (Navarrete-Bolaños et al., 2005). Nevertheless, it should be emphasized that the pro-oxidant activity of lutein, esterified with polyunsaturated fatty acids have been concerned in order to avoid unfavorable effect for human health (Pérez-Gálvez and Mínguez-Mosquera, 2005; Subagio and Morita, 2001). As of the results, lutein should be replaced with unesterified lutein. Currently, there is a lack of commercial available sources of unesterified lutein for food and pharmaceutical products. However, evidence from this study was remarkable that yellow silk cocoons were found as a novel source of lutein. More than 90% of free lutein and its isomers were extracted from degummed cocoons without further purification. Despite their total lutein content existing in a lower amount than that found in marigold petals, because of low impurities resulting in the simplicity of extraction and preparation processes. Therefore, it was noteworthy that yellow silk cocoons were probably emerging into a food market as a novel particular source of lutein for food supplemental and nutraceutical products in this decade.

4.3 HPLC analysis of carotenoids in yellow silk cocoons

4.3.1 Separation and identification of carotenoids

Reversed-phase system was performed in this study, since several disadvantages as lower column stability, a poorer reproducibility of the retention times, and a longer time required for column equilibration were found in normal-phase system. The analyses were conducted with conditions that prevented carotenoids from the loss in the concentration during HPLC analysis, as described by Feltl et al. (2005). By using C_{18}-bound phase, good separation was achieved within 40 min. The pigments were identified by comparing the order of elution, retention time, and absorption maxima against carotenoid standards and the literature data.

Figure 4.3 shows the chromatograms of the pigment extracted with various extracting solvents. It was clearly that similar profiles of carotenoid distribution were found among the lutein extracts according to the UV/VIS absorption spectra obtained using spectrophotometer. Figure 4.3A shows a typical chromatogram of the mixture of standards. The standard curves for lutein showed linearity with coefficient of correlation of 0.998. With this HPLC condition, the more polar xanthophylls such as lutein and its isomers eluted before hydrocarbon carotenoids such as α- and β-carotenes. The peaks identified in this study were completely eluted under isocratic HPLC condition during early seven minutes. The gradient elution was then performed after 20 min until completely in order to separation of others existing carotenoids with none or less polarity, including α-carotene, β-carotene and xanthophyll esters. It was emphasized that the lutein extracts from yellow silk cocoons showed a carotenoid pattern in major peak of lutein (Figure 4.3B) similar to standard peak and previous reports (Jouni and Wells, 11996; Kimura et al., 2007; Tsao and Yang, 2006). Lutein in

the extracts was identified at a retention time of 6.01-6.02 min with absorbance maxima at 445 nm. This peak was confirmed by comparison with the peak of lutein standard. Apart from this major carotenoid, its stereoisomer as zeaxanthin and (*Z*)-geometric isomers, obviously (9-*Z*), (9'-*Z*), (13-*Z*), and (13'-*Z*)-isomers were tentatively identified in accordance with elution order from literatures (Lakshminarayana et al., 2008; Tsao et al., 2004; Tsao and Yang, 2006; Granado et al., 2001). It is interesting that the pigment extracted from yellow silk cocoons yield mainly lutein without detectable impurity from other carotenoids. From these results, it should be noted that this may be the first report of carotenoid extraction from yellow silk cocoons which found as a new rich source of lutein with low impurities.

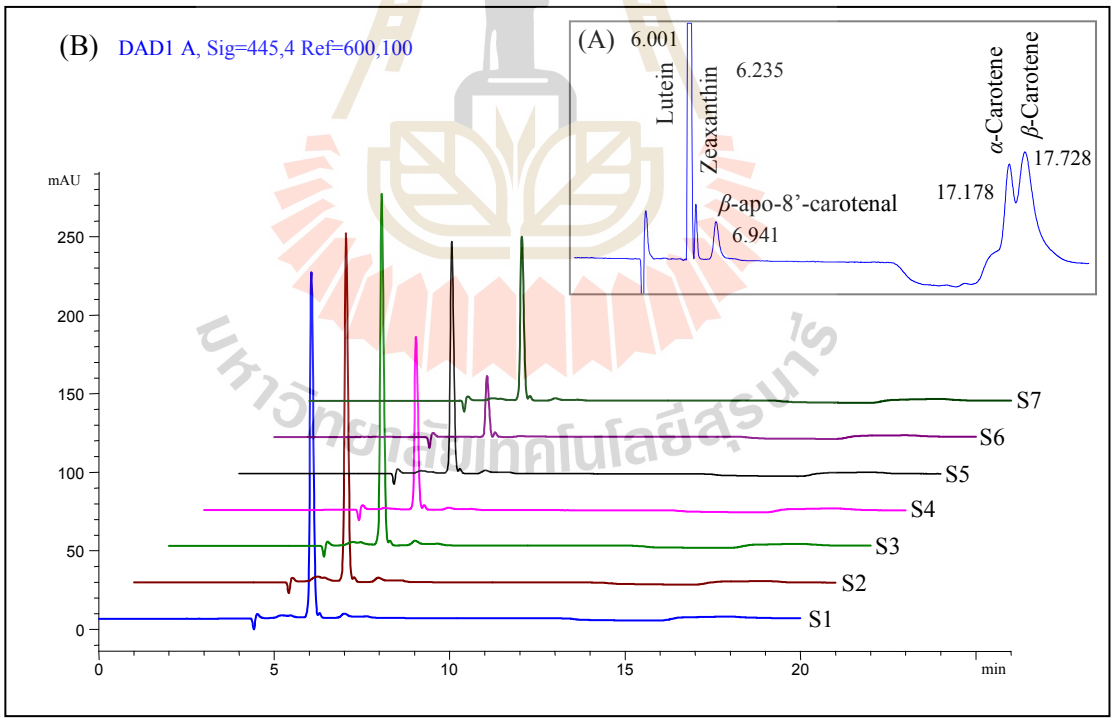

Figure 4.3 Carotenoid profiles of (A) standard carotenoids and (B) pigment extracts from yellow silk cocoons, degummed at 121ºC for 15 min and extracted with various solvent systems, S1-S7, as described in text.

Table 4.6 Distribution and retention time of carotenoid compositions in pigments extracts from yellow silk cocoons extracting with various solvent systems.

Sample		Distribution of carotenoids [a]					
		(13-*Z*) lutein[b]	(13'-*Z*) lutein[c]	(all-*E*) lutein[a]	(all-*E*) zeaxanthin[bc]	(9-*Z*) lutein[b]	(9'-*Z*) lutein[bc]
Retention time (min)	S1	5.187[b] ±0.011	5.423[a] ±0.001	6.025[a] ±0.002	6.256[a] ±0.002	6.962[a] ±0.002	7.618[a] ±0.006
	S2	5.214[a] ±0.003	5.399[b] ±0.011	6.013[a] ±0.008	6.245[a] ±0.009	6.942[a] ±0.022	7.596[a] ±0.025
	S3	5.211[a] ±0.009	5.415[ab] ±0.011	6.019[a] ±0.008	6.251[a] ±0.008	6.961[a] ±0.021	7.617[a] ±0.018
	S4	5.135[c] ±0.010	-	6.014[a] ±0.010	6.244[a] ±0.010	6.964[a] ±0.026	7.612[a] ±0.030
	S5	5.145[c] ±0.006	-	6.016[a] ±0.006	6.246[a] ±0.006	6.966[a] ±0.016	7.613[a] ±0.017
	S6	5.121[d] ±0.008	-	6.016[a] ±0.003	6.248[a] ±0.003	6.963[a] ±0.006	-
	S7	5.224[a] ±0.003	5.411[ab] ±0.003	6.017[a] ±0.002	6.244[a] ±0.003	6.968[a] ±0.004	7.615[a] ±0.005
Relative abundance (%)	S1	2.11[bcd] ±0.33	1.47[b] ±0.27	92.87[a] ±0.28	1.15[cd] ±0.13	1.88[b] ±0.08	0.52[c] ±0.08
	S2	3.35[a] ±0.40	1.89[a] ±0.01	91.31[b] ±0.51	1.00[d] ±0.13	1.95[b] ±0.05	0.50[c] ±0.03
	S3	2.81[ab] ±0.77	1.74[ab] ±0.09	91.87[b] ±0.92	1.05[d] ±0.13	1.99[b] ±0.06	0.55[bc] ±0.03
	S4	2.04[abc] ±0.13	-	93.21[a] ±0.12	2.01[b] ±0.11	2.03[b] ±0.04	0.71[a] ±0.05
	S5	2.19[bcd] ±0.29	-	93.50[a] ±0.21	1.57[bc] ±0.12	2.08[b] ±0.05	0.66[a] ±0.03
	S6	1.73[d] ±0.05	-	87.99[c] ±0.29	5.10[a] ±0.18	5.18[a] ±0.42	-
	S7	2.69[abc] ±0.11	1.74[ab] ±0.29	91.26[b] ±0.54	1.59[bc] ±0.56	2.08[b] ±0.01	0.64[ab] ±0.08

Note: [a] Average of triplicate determinations ± standard deviation. Values were obtained with C18-RP column, and photodiode array detection at 445 nm. HPLC condition was described in text. Different letters on the same column indicate significant differences ($p \leq 0.05$).

The distribution and retention time of carotenoids comprised in the pigment extracts were given in Table 4.6. The results showed good reproducibility of the retention time even for the peaks that were not well resolved. There was no significant difference on the retention time of (all-*E*)-lutein among the pigment extracts ($p>0.05$), similarly to (all-*Z*)-zeaxanthin, (9-*Z*)-lutein, and (9'-*Z*)-lutein. In contrast to those compositions, a slight variation can be seen in the retention times of the (13-*Z*) and (13'-*Z*)-isomers of lutein. This result was possibly associated with their low relative quantity that, consequently, diminished their hydrophobic interaction on the stationary phase (Lin and Chen, 2003). The unresolved peaks of (13'-*Z*)-lutein and, occasionally,

(9'-*Z*)-lutein were obtained for samples of the pigment extracted with none or less polar solvents (S4, S5, and S6). As a result of the lowest amount of lutein obtained by the utilization of these extracting solvent, thereby, its (13'-*Z*) and (9'-*Z*)-isomers probably existed in a very low relative quantity. Considering that these compositions possibly co-eluted with (13-*Z*) and (9-*Z*)-isomers, respectively, since only a number or whether arrangement of the double bonds was different between these geometric isomers (Rodriguez-Amaya, 2001).

The extraction efficiency of various solvent systems was also compared based on carotenoid distribution. Apart from (13'-*Z*)-lutein, the utilization of seven extracting solvents resulted in significant difference on the relative amount of (all-*E*)-lutein, (all-*E*)-zeaxnthin, (13-*Z*)-lutein, (9-*Z*)-lutein, and (9'-*Z*)-lutein ($p<0.05$). The highest distribution of (all-*E*)-lutein about 92.9-93.5% was found when the pigment was extracted with hexane/ethanol (S1), hexane/ethyl acetate (S4), and ethyl acetate (S5). By the use of hexane, the lowest relative amount of 88.0% was found for lutein. Whereas, the greater amount of lutein (91.3-91.9%) were extracted with the combination of hexane and a particular ratio of organic solvents with higher polarity such as hexane/acetone (S2), hexane/acetone/ethanol (S3), and hexane/ethanol/ethyl acetate (S7). In addition to lutein, a small amount of its stereoisomer, zeaxanthin, and their (13-*Z*), (13'-*Z*), (9-*Z*) and (9'-*Z*)-isomers were also detected. These observed isomers pointed to the isomerization of lutein that possibly occurred during the extraction process. It was also emphasized that the extracting solvents used in this study did not largely affect on the degradation and the isomerization of lutein. Evidences suggested that acetone was frequently involved in a solvent for extraction of carotenoid from high moisture sources such as vegetables, fruits, and

microorganisms (Kimura and Rodriguez-Amaya, 2002; Tsao et al., 2004; Rouseff and Hofsommer, 1996; Updike and Schwartz, 2003). Thus, to keep carotenoids in the acetone solution can lead to their substantial degradation (Craft and Soares, 1992; Rodriguez-Amaya, 2001). However, as found in this study, there was negligible effect on the rearrangement of lutein to either (9-*Z*)-configurations or zeaxanthin by using hexane/acetone (5:3, v/v) or hexane/acetone/ethanol (3:2:1, v/v/v) as an extracting solvents. Whereas, the (13-*Z*)-configurations were likely increased with increase in a ratio of acetone. However, those (13-*Z*) and (13'-*Z*)-isomers were not significantly different among the use of hexane/acetone/ethanol (3:1:2, v/v) and other solvent systems (p>0.05). Moreover, insignificant difference was also observed on the relative amount of (9-Z)-lutein in the extracts obtained using other acetone-free solvent systems (p<0.05). Excepted for using hexane (S6) as an extracting solvent that the highest amount of (9-*Z*)-lutein separated under the C_{18} chromatographic column probably attributed to the co-elution of (9'-*Z*)-lutein.

The effect of thermal treatment on the isomerization of lutein as well as other carotenoids has been well established (Aman et al., 2005; Aman, Schieber and Carle, 2005; Milanowska and Gruszecki, 2005; Schieber and Carle, 2005; Tang and Chen, 2000; Updike and Schwartz, 2003). In this study, the heat-induced isomerization of lutein was believed to be a major cause of the rearrangement of lutein, since thermal treatment was involved in the extraction process. The formation of small amount of (9-*Z*) and (13-*Z*)-geometric isomers as well as their relative isomers was found in accordance with previous reports that the effect of thermal process was extensively described (Aman et al., 2005; Updike and Schwartz, 2003). Previous studies also proved that these isomers of lutein have been preferably occurred at either the 9- or

13-position depending on the physical states or food matrixes (Aman, Schieber and Carle, 2005; Chandler and Schwartz, 1987; Marx, Schieber and Carle, 2003; Pott et al., 2003). Therefore, it can be proposed that the distribution of (*Z*)-geometric isomers of lutein found in the lutein extracts was possible owing to thermal isomerization.

4.3.2 Separation of standard lutein and zeaxanthin sterioisomers

Lutein and zeaxanthin were different in the position of one double bond in the ionone ring. Likewise in their (Z)-geometric isomers that only the arrangement of one conjugated double bond was dissimilar. Despite a small difference in their chemical structures, it definitely affected on the resolution of chromatographic separation. The C_{18} stationary phase was previously used for numerous separations and thereby for carotenoid separation (Aman et al., 2005) nevertheless, they might be inadequate to provide the best resolution for carotenoids with similar structure. Therefore, in some cases that (*Z*)-isomers were not well resolved, a tailing shoulder can be seen after a peak of their parent xanthophylls or between relative isomers such as (9'-*Z*) to (9-*Z*) and (13'-*Z*) to (13-*Z*)-isomer, or vice versa. During the past decade, C_{30} stationary phase has been emerged to be the best choice for the separation of carotenoids with similar structure. Previous studies revealed the advantages of this chromatographic column, particularly, an improvement of shape selectivity and increase in the retention time that consequently achieved the separation between (all-*E*)-lutein and (all-*E*)-zeaxanthin (Aman et al., 2005; Rouseff and Hofsommer, 1996).

In order to compare the resolution on the C_{18} and C_{30} stationary phases in this study, the chromatographic separation of standard lutein and its isomers was carried out with the same operating conditions. The capacity ratio (*k'*) and selectivity (α) of the separation were investigated as given in Table 4.7. Under the C_{30} column, the

resolution of lutein and its isomers was improved after adjusting solvent systems, flow rates, and column operating temperatures. The retention of (all-*E*)-lutein for 6.1 min on the C_{18} column was increased to 24.5 min when the C_{30} stationary phase was replaced. This prolonged retention times was similarly observed for its isomers that, consequently, contributed to increase in the *k'* values. A gradient system of acetronitrile, methanol and ethyl acetate displayed a good selectivity characteristic (α = 1.18-1.19) for (all-*E*)-lutein and provided partial resolution for its (*Z*)-isomers. With this column, the (13-*Z*) and (13'-*Z*)-lutein were well resolved, in spite of presence in a small amount. Although the separation was manipulated using the same mobile phase and operating conditions, different elution patterns were obtained between these two columns. These results indicated that lutein and its isomers displayed good affinity on a polymeric C_{30} column and by mean of which their resolution of the separation was improved.

Figure 4.4 shows the chromatogram of standard (all-*E*)-lutein and it isomerized products. It was clearly that light exposure at 1,500 Luk for 30 min provoked the isomerization of lutein and zeaxanthin to their (*Z*)-geometric isomers, predominantly the (9-*Z*) and (9'-*Z*)-isomers. After iodine-catalyzed photoisomerization of (all-*E*)-lutein five predominant peaks were observed, while the isomerization of (all-*E*)-zeaxanthin resulted in three major peaks. These obtained results well agreed with the study of Aman and co-workers (2005). Moreover, it is interesting to note that (all-*E*)-lutein and (all-*E*)-zeaxanthin were preferably rearranged to the predominant (9-*Z*) and (13-*Z*)-geometric isomers, in accordance with heat-induced isomerization (Chandler and Schwartz, 1987; Milanowska and Gruszecki, 2005; Updike and Schwartz, 2003).

Table 4.7 Retention time, concentration, relative abundance, capacity ratio (*k'*) and selectivity (α) of standard xanthophylls and (*Z*)-geometric isomers separated under the C_{18} and C_{30} RP-columns with the same solvent system.

Column	Sample [d]	UK_1 [a]	(13-*Z*) lutein	(13'-*Z*) lutein	(all-*E*) lutein	(all-*E*) zeaxanthin	(9-*Z*) lutein	UK_2 [a]	(9'-*Z*) lutein	(9-*Z*) zeaxanthin
Retention time (min)										
C_{30}	L-Dark	-	19.847	21.501	24.524	26.474	31.121	-	-	-
	L-Light	-	19.849	21.647	24.556	26.522	31.334	-	-	-
	L-Iodine	18.338	19.566	21.246	24.059	26.532	29.985	32.311	36.013	38.744
C_{18}	L-Dark	-	-	-	6.105	6.355	7.031	-	-	-
	L-Light	-	-	-	6.095	6.346	7.031	-	-	-
	L-Iodine	-	-	-	6.112	6.396	7.064	-	8.364	-
Relative abundance (%)										
C_{30}	L-Dark	-	0.658	0.444	87.136	10.349	1.412	-	-	-
	L-Light	-	0.781	0.536	86.692	10.643	1.349	-	-	-
	L-Iodine	2.654	7.249	7.495	53.464	7.784	9.567	1.239	9.176	1.371
C_{18}	L-Dark	-	-	-	95.081	1.160	3.259 [e]	-	-	-
	L-Light	-	-	-	94.251	2.386	3.362 [e]	-	-	-
	L-Iodine	-	-	-	92.708	4.212	1.097	-	1.984	-
Capacity ratio [b]		k'_1	k'_2	k'_3	k'_4	k'_5	k'_6	k'_7	k'_8	k'_9
C_{30}	L-Dark	-	2.703	3.012	3.576	3.940	4.807	-	-	-
	L-Light	-	2.704	3.039	3.582	3.949	4.847	-	-	-
	L-Iodine	2.423	2.652	2.966	3.491	3.953	4.597	5.032	5.723	6.232
C_{18}	L-Dark	-	-	-	0.425	0.484	0.642	-	-	-
	L-Light	-	-	-	0.423	0.482	0.642	-	-	-
	L-Iodine	-	-	-	0.426	0.493	0.649	-	0.952	-
Selectivity [c]		-	$\alpha_{2/1}$	$\alpha_{3/2}$	$\alpha_{4/3}$	$\alpha_{5/4}$	$\alpha_{6/5}$	$\alpha_{7/6}$	$\alpha_{8/7}$	$\alpha_{9/8}$
C_{30}	L-Dark	-	-	1.114	1.187	1.102	1.220	-	-	-
	L-Light	-	-	1.124	1.179	1.102	1.227	-	-	-
	L-Iodine	-	1.095	1.118	1.177	1.132	1.163	1.094	1.137	1.089
C_{18}	L-Dark	-	-	-	-	1.137	1.326	-	-	-
	L-Light	-	-	-	-	1.139	1.332	-	-	-
	L-Iodine	-	-	-	-	1.155	1.316	-	1.468	-

Note: [a] $UK_{1\text{-}2}$ represents unknown peaks with unidentified. [b] The number subscribed on capacity ration (*k'*) refers to each identified xanthophylls in the same column. [c] Selectivity with subscribed divided number represents the efficiency of a chromatographic system on the separation between two adjacent peaks of xanthophylls that is calculated from an equation: $\alpha = k'_{\text{behind-peak}}/k'_{\text{earlier-peak}}$. [d] Standard lutein samples were prepared (L-Dark) without isomerization, and with (L-Light) light and (L-Iodine) iodine isomerization. [e] Peaks were unresolved between (9-*Z*) and (9'-*Z*)-isomers.

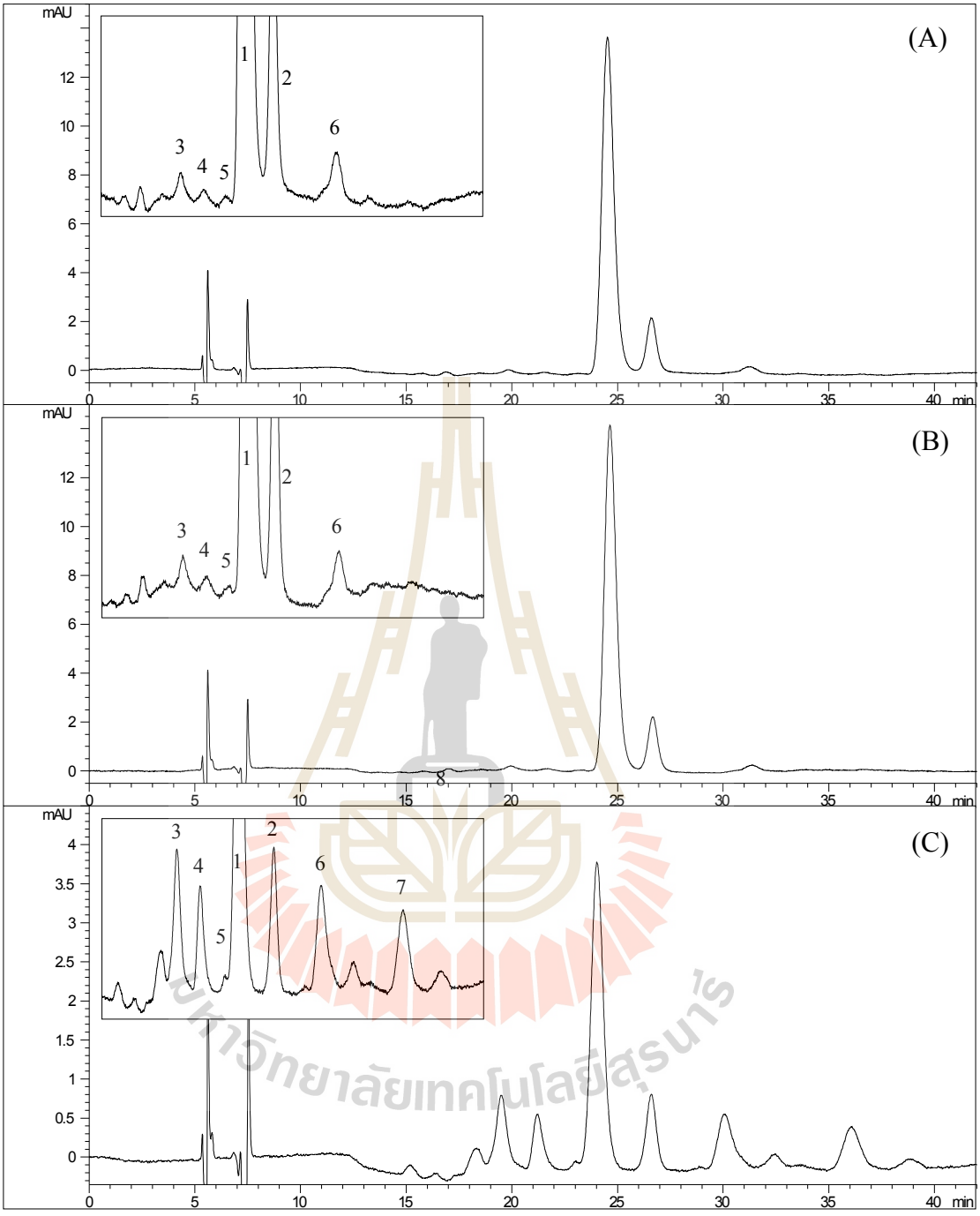

Figure 4.4 C_{30}-RP HPLC chromatograms of (A) standard lutein and carotenes and their isomerized products by fluorescence light (1,500 Lux) (C) with and (B) without iodine isomerization. Peaks: 1 = (all-*E*)-lutein; 2 = (all-*E*)-zeaxanthin; 3 = (13-*Z*)-lutein; 4 = (13'-*Z*)-lutein; 5 = (13-*Z*)-zeaxanthin; 6 = (9-*Z*)-lutein; 7 = (9'-*Z*)-lutein; and 8 = (9-*Z*)-zeaxanthin.

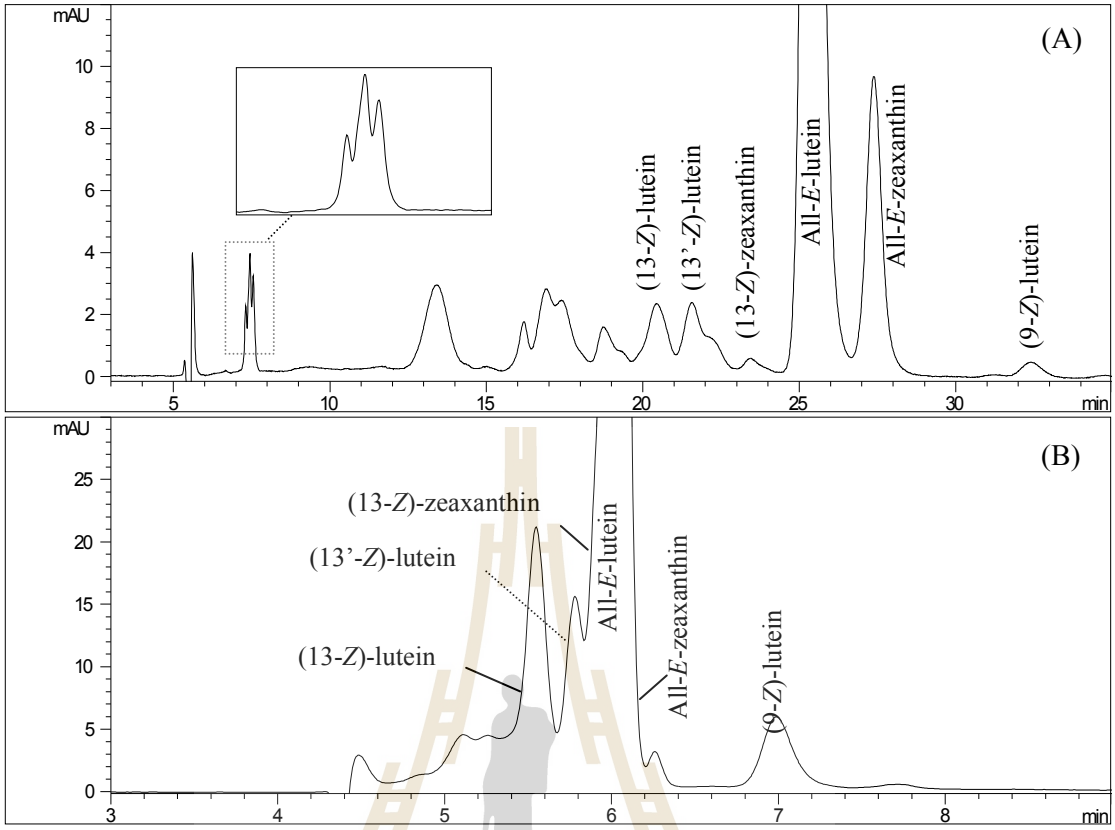

Figure 4.5 Carotenoid distribution of pigment extract from yellow silk cocoons, degummed at 85ºC for 30 min, reversed-phase chromatographic separated using (A) C_{30} and (B) C_{18} columns.

4.3.3 Separation of lutein and its isomers in the pigment extracts

Lutein and zeaxanthin stereoisomers in the lutein extracts were identified in accordance with the elution order of (all-*E*)-lutein with thermal isomerized products (Dachtler et al., 2001; Emenhiser et al., 1996; Emenhiser, Sander and Schwartz, 1995; Updike and Schwartz, 2003). Figure 4.5 and 4.6 show the elution profiles of lutein and its isomers extracted from yellow silk cocoons after degumming at 85ºC for 30 min, and 121ºC for 15 min. Apart from predominant (all-*E*)-lutein and (all-*E*)-zeaxanthin in the extract, (9-*Z*) and (13-*Z*)-isomers were detected with (13-*Z*) and

(13'-*Z*)-lutein being present in higher amount. The lutein isomers were not well resolved under the C_{18} column (Figure 4.5B). Consequently, the tentative identification of (all-*E*)-lutein was possibly over estimated around 7.1%, since its actual amount was relatively compensated with (13-*Z*)-lutein, (13'-*Z*)-lutein, and, occasionally, (9-*Z*)-lutein and (all-*E*)-zeaxanthin. In contrast to the C_{30} column, major isomeric components were more clearly separated (Figure 4.5A). Evidence from this study showed that the C_{18} column was appropriately for routine analysis of lutein, whereas the separation of it isomers could be achieved by mean of the C_{30} column.

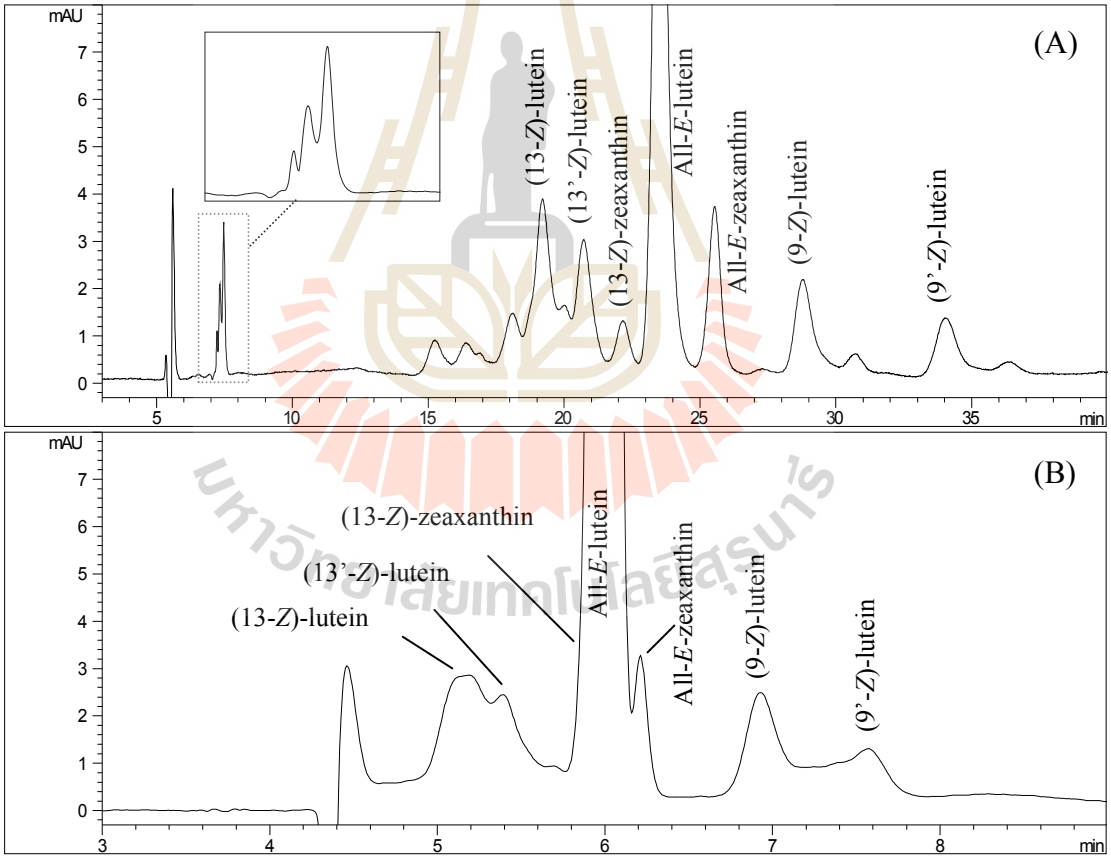

Figure 4.6 Carotenoid distribution of pigment extract from yellow silk cocoons, degummed at 121ºC for 15 min, reversed-phase chromatographic separated using (A) C_{30} and (B) C_{18} columns.

Regarding to previous results, yellow silk cocoons degumming at 121°C for 15 min were used for the extraction of lutein for further analysis. The elution profiles of the extract were shown in Figure 4.6. It is interesting that obtained pigment gave the separation pattern in accordance with previous standard isomerization. The influence of thermal treatment in the degumming process promoted the presence of (13-*Z*)-isomers (8.37%) with unidentified (*Z*)-isomers (9.47%) that bound loosely to the stationary phase. The (9-*Z*) and (9'-*Z*)-isomers in the extract (6.08 and 4.67%) present in a lower amount than that found in the light-induced isomerization of (all-*E*)-lutein (9.57 and 9.18%). Remarkably, the predominant (*Z*)-isomers of lutein in the pigment extracts were (13-*Z*) and (13'-*Z*)-isomers. This result was harmonized with the study of Updike and Schwartz (2003) that the effect of thermal processing of vegetables on the *E*/Z isomerization of (all-*E*)-lutein was demonstrated. However, the (*Z*)-isomers of lutein probably existed in various proportion depending on the physiological and biochemical changes as well as heating conditions (Aman, Schieber and Carle, 2005; Schieber and Carle, 2005; Updike and Schwartz, 2003).

4.3.4 The absorption characteristic of the lutein extracts

The photodiode array spectra of lutein from yellow silk cocoons were illustrated in Figure 4.7. In this study, the maximum spectrum without shifting was found in purified carotenoids. For (all-*E*)-lutein, its visible spectra were closely to literature values that the λ_{max} values at 421-422, 444-445 and 473-474 nm were reported (Chen and Chen, 1993; Rodrigures-Amaya, 2001; Wrolstad et al., 2005). In case of the stereoisomer and (*Z*)-geometric isomers of lutein, the λ_{max} values normally shifted from that absorbed by (all-*E*)-lutein to an either longer or shorter wavelength. For instance, zeaxanthin and (9-*Z*)-lutein their λ_{max} values shifted to be 428, 450, 478

nm, and 418, 443 and 470 nm, respectively (Chen and Chen, 1993; Rodrigures-Amaya, 2001). The maximum absorption bands of the pigment extracts were disagreed with that provided by other common carotenoids, such as *β*-carotene (425, 450 and 478 nm), *α*-carotene (423, 444 and 473 nm), and *β*-cryptoxanthin (428, 450 and 478 nm) (Craft and Soares, 1992; Rodrigures-Amaya, 2001). Obtained results of the absorption spectra proved that the pigment composition in the silk cocoons resembled to (all-*E*)-lutein and its isomers.

Table 4.8 shows the λ_{max} values of (all-*E*)-lutein comprising in the extracts from degummed cocoons and pigmented-sericin solutions. With the mobile phase used in this study, the variation of the λ_{max} values for any carotenoids was considerably minimized (Britton, Liaaen-Jensen and Pfander, 2004). As a result of thermal-induced isomerization, the λ_{max} values with around 3 nm of the hypsochromic shift was observed for lutein in the extracts. From the cocoons degumming at 85ºC for 30 min the lutein extract showed the λ_{max} values at 447 and 476 nm, whereas at 121ºC for 120 min the values were displaced to be 443 and 471 nm. The photodiode array spectra of lutein scanning from 290 to 600 nm shown *cis* absorption peak , or namely *cis*-peak, in the ultraviolet region with maximum wavelength at around 333-334 nm (Figure 4.7). This absorption band was distinctively observed in the lutein extract from the cocoons, degummed at 121ºC. Schieber and Carle (2005) suggested that this absorption characteristic associated with the localization of *cis*-double bond that shows the greatest absorption intensity when the double bond was located near or at the center of the chromophore. As a result of the interference of the *cis*-peak, the maximum absorption of (all-*E*)-lutein consequently shifted to the lower wavelengths. These results were sustainable for the separation of (all-*E*)-lutein with partial co-elution of its (*Z*)-isomers under the C_{18} chromatographic condition.

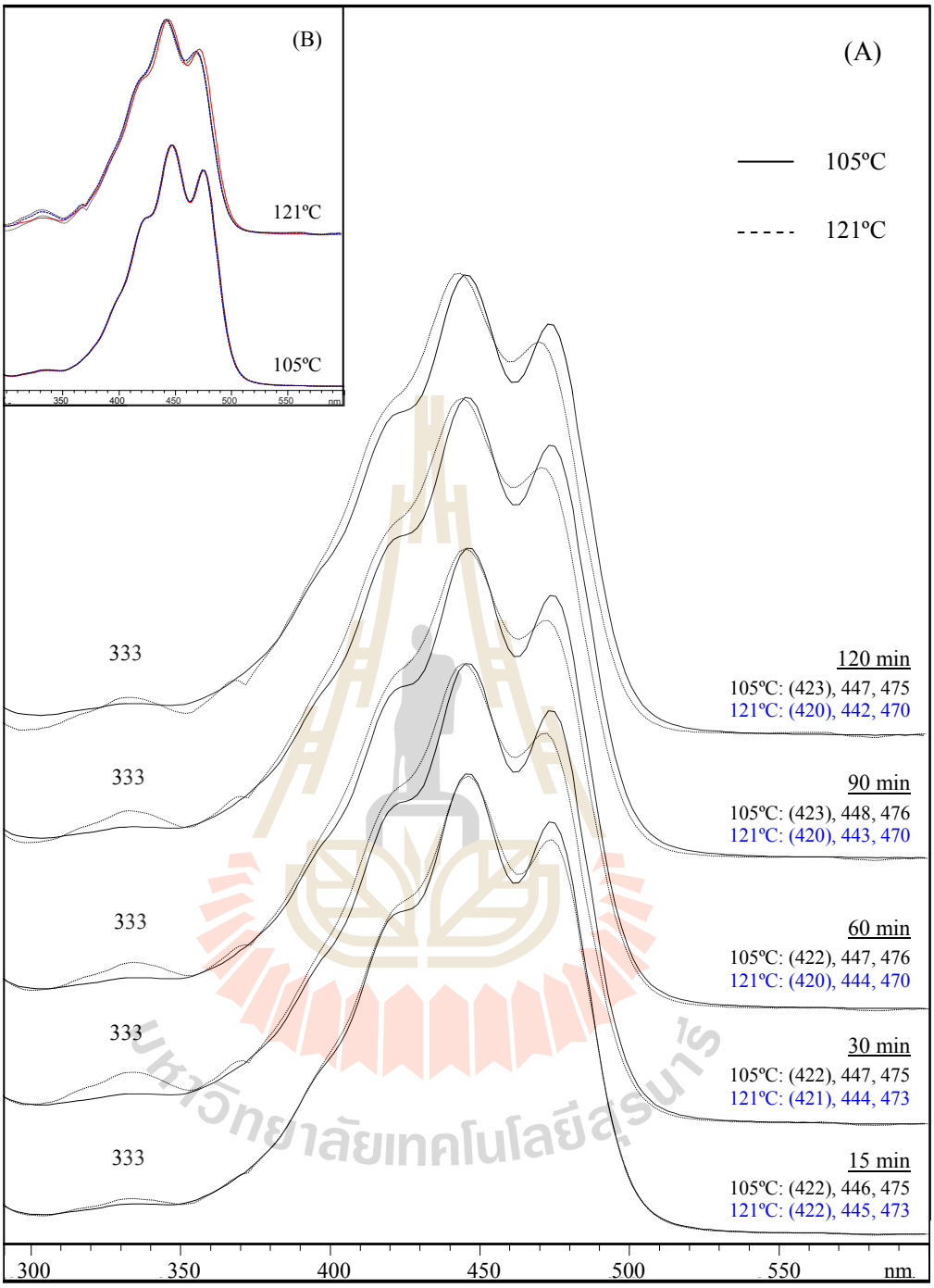

Figure 4.7 Photodiode array spectra of lutein from yellow silk cocoons, separated under the C_{18} chromatographic condition with monitored at 445 nm.

Table 4.8 Absorption maxima of lutein from yellow silk cocoons separated under reversed-phase chromatographic condition and the literature data.

Sample	Degummed cocoons			Degumming solution			Literature value					
	λmax (nm)			λmax (nm)			λmax (nm)			Solvent	Source	Ref.
	I	II	III	I	II	III	I	II	III			
STD	(423)	446	474	-	-	-	422	445	474	Ethanol	STD	(1)
							422	445	474	Ethanol	STD	(2)
C1	420	442	469	-	-	-	(423)	445	474	Ethanol	Human retina	(18)
C2	424	445	469	-	-	-	421	445	473	Ethanol	Cooked sweet potato leaves	(21)
C3	423	444	471	-	-	-	(426)	448	472	Ethanol	Flower of daylily	(2)
							424	444	472	Hexane	STD	(2)
S1	(420)	446	471	-	-	-	422	444	473	Hexane	STD	(3)
S2	(420)	444	471	-	-	-	(424)	448	476	Eluent-1	Human retina: (all-*E*)-lutein	(14)
S3	(419)	443	472	-	-	-	(420)	442	470	Eluent-1	(9-*Z*)-lutein	(14)
S4	(419)	443	471	-	-	-	(420)	444	472	Eluent-1	(9'-*Z*)-lutein	(14)
S5	(419)	444	473	-	-	-	(418)	440	468	Eluent-1	(13-*Z*)-, (13'-*Z*)-lutein	(14)
S6	(419)	444	472	-	-	-	424	446	474	Eluent-2	STD	(6)
S7	(419)	444	471	-	-	-	-	442	-	Eluent-2	STD	(7)
							-	444	473	Eluent-2	Canned/Microwaved vegetables	(13)
A30	(420)	447	476	-	-	-	-	446	474	Eluent-2	Dry maize	(15)
A60	(419)	446	474	-	-	-	(423)	444	472	Eluent-3	STD	(5)
A90	(420)	446	476	-	-	-	425	445	473	Eluent-4	STD	(2)
A120	(420)	446	476	-	-	-	417	441	465	Eluent-5	Human serum	(4)
B15	(420)	447	475	(422)	446	475	(424)	444	470	Eluent-6	Carrot juice	(19)
B30	(419)	446	475	(422)	447	475	-	447	475	Eluent-7	Dry maise	(15)
B60	(419)	446	474	(422)	447	476	424	445	474	Eluent-7	Taiwanes orange peels	(20)
B90	(418)	446	474	(423)	448	476	423	445	474	Eluent-8	Cooked sweet potato leaves	(21)
B120	(418)	445	473	(423)	447	475	421	445	474	Eluent-7	Orange juice	(8)
C15	(419)	444	472	(422)	445	473	(424)	444	470	Eluent-9	Orange juice	(16)
C30	(419)	444	472	(421)	444	473	(425)	446	472	Eluent-10	Orange juice	(17)
C60	(419)	443	472	(420)	444	470	422	446	476	Eluent-11	Tomato juice	(9)
C90	(418)	444	472	(420)	443	470	416	438	468	Eluent-10	Flower of dandelion	(10)
C120	(418)	443	471	(420)	442	470	-	444	473	Eluent-12	Flower of marigold	(11)
							-	448	475	Eluent-9	Lutein ester	(12)

Note: S1-7 represents degummed cocoons, extracted with various solvent systems, as described in text. Raw silk cocoons were extracted of pigments at room temperature for 6 hours under (C1) dark and (C2) light and for (C3) 48 hours under dark. A, B, and C with suffix number represent degumming temperature at 85, 105 and 121ºC, respectively, for various time (min). Solvent system for each eluent was used by,
(Eluent-1) hexane/DCM/MeOH/DIPEA, [14] Khachik, Bernstein and Garland (1997);
(Eluent-2) MTBE/MeOH, [6] Breithaupt (2004), [7] Emenhiser, Sander and Schwartz (1995), [13] Updike and Schwartz (2003), and [15] Kimura et al. (2007);
(Eluent-3) MTBE/MeOH/water, [5] Aman et al. (2005);
(Eluent-4) MTBE/methylene chloride, [2] Tai and Chen (2000);
(Eluent-5) MeOH/methylene chloride, [4] Rajendran, Pu and Chen (2005);
(Eluent-6) ACN/MeOH/methylene chloride, [19] Chen, Peng and Chen (1996);
(Eluent-7) ACN/MeOH/ethyl acetate, [8] Gama and Sylos (2007), [15], and [20] Yen and Chen (1995);
(Eluent-8) ACN/MeOH/chloroform/hexane, [21] Chen and Chen (1993);
(Eluent-9) MeOH/TBME, [12] Burns, Fraser and Bramley (2003) and [16] Corts et al. (2004);
(Eluent-10) MeOH/MTBE/water, [10] Meléndez-Martínez et al. (2006) and [17] Rouseff and Hofsommer (1996);
(Eluent-11) 1-Butanol/ACN/MTBE, [9] Lin and Chen (2003); and
(Eluent-12) Hexane/ethyl acetate, [11] Hadden et al. (1999).
Absorption maxima in ethanol were measured by [1] Rodriguez-Amaya (2001), [2], [18] Landrum and Bone (2001), and [21], additionally, in hexane were measure by [2] and [3] Breithaupt (2004).

4.3.5 Quantitative distribution of lutein isomers in the pigment extracts

Figure 4.8 and 4.9 show the C_{30}-reversed phase HPLC separation of lutein and its isomers in the degummed cocoons and pigmented-sericin solutions from various degumming temperatures. There were four stereoisomers of (all-*E*)-lutein with two (*Z*)-geometric isomers of (all-*E*)-zeaxanthin detected during elution period. Because of the inadequate data of identification and structural elucidation, unfortunately, there were some of unknown *cis* peaks and polar fractions without characterized. Unknown *cis* peaks, early eluted before (13-*Z*)-lutein were observed for degumming temperature at 85ºC. However, these peaks appeared minimally at higher temperatures, while increase in the relative amount of predominant (*Z*)-isomers were observed instead. It was considered that these peaks may represent either (*Z*)-isomers of lutein or its epoxides, since the (*Z*)-isomers were susceptible to the oxidation due to severe heating condition (Chen and Chen, 1993; Gama and Sylos, 2007; Rodriguez-Amaya and Kimura, 2004). In general, oxidative degradation is a principal cause of extensive loss of carotenoids that depends on the availability of oxygen and the acceleration of light, enzymes, metals, and co-oxidation with lipid hydroperoxides (Rodriguez-Amaya, 2001). In this study, the oxidative degradation of lutein isomers possibly took place in the lutein extracts as a result of the attendance of atmospheric oxygen and light. However, from the facts that the formation of carotenoid epoxides is naturally accompanied with the biosynthetic partway, mostly in some plants (Deli et al., 2001; Matus, Deli and Szabolcs, 1991; Meléndez-Martínez et al., 2005; Razungles et al., 1996; Rodriguez-Amaya and Kimura, 2004), therefore further study is needed for the identification and structural elucidation of lutein epoxides.

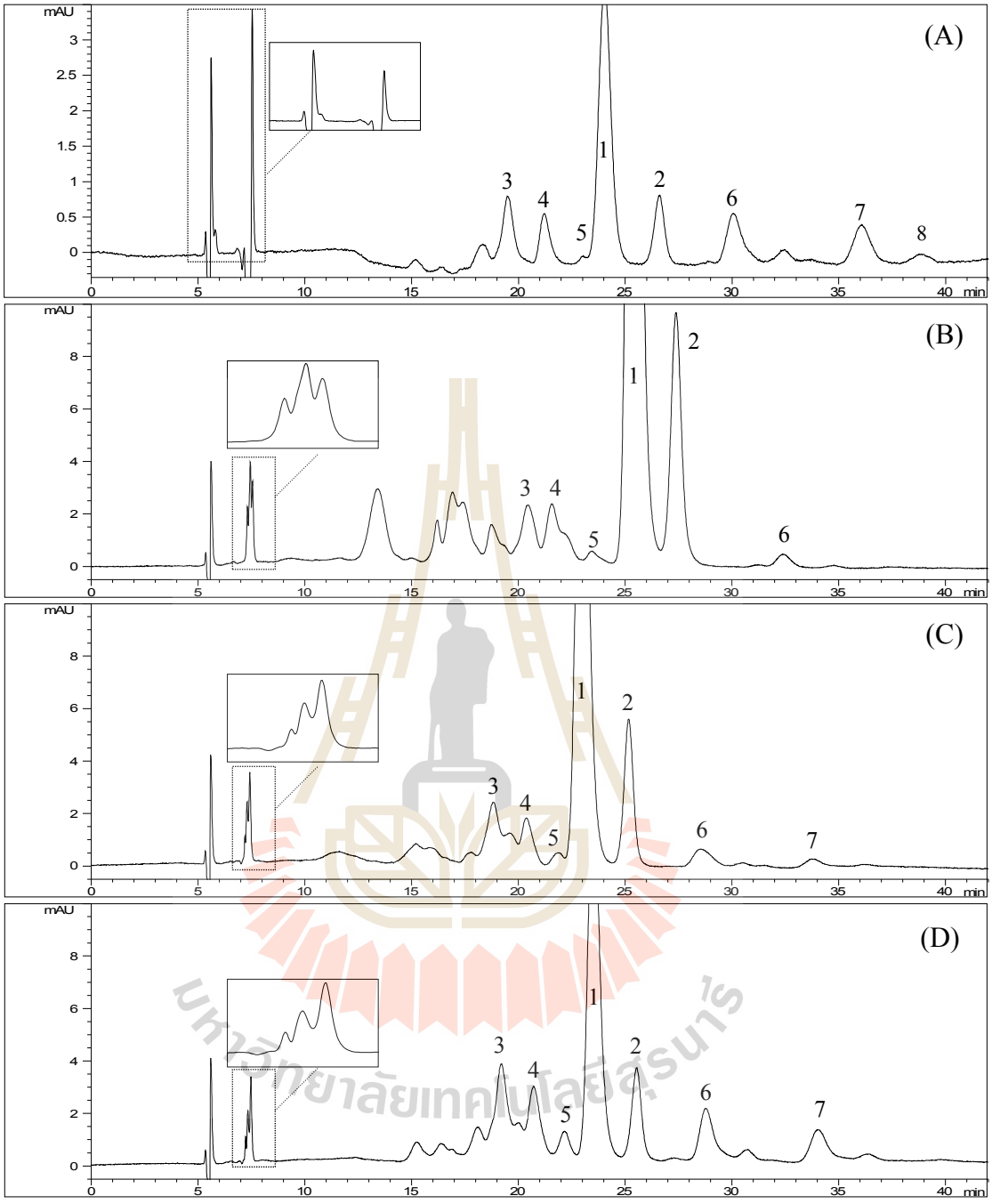

Figure 4.8 C_{30}-RP HPLC chromatograms of (A) standard lutein and pigment extracts from yellow silk cocoons, degummed at (B) 85ºC, (C) 105ºC, and (D) 121ºC for 30 min. Peaks: 1 = (all-*E*)-lutein; 2 = (all-*E*)-zeaxanthin; 3 = (13-*Z*)-lutein; 4 = (13'-*Z*)-lutein; 5 = (13-*Z*)-zeaxanthin; 6 = (9-*Z*)-lutein; 7 = (9'-*Z*)-lutein; and 8 = (9-*Z*)-zeaxanthin.

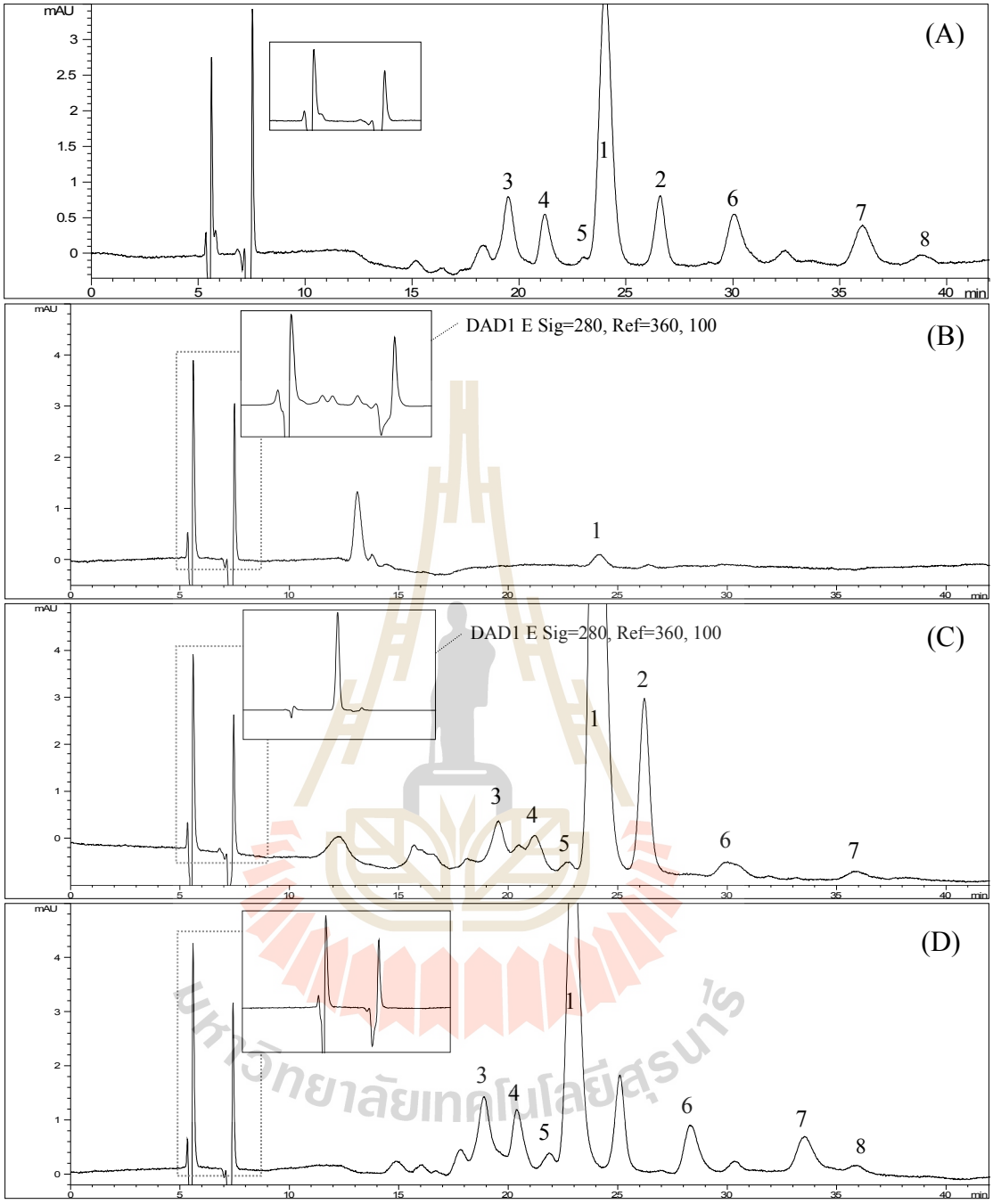

Figure 4.9 C_{30}-RP HPLC chromatograms of (A) standard lutein and pigment extracts from pigmented-sericin solutions, degummed at (B) 85ºC, (C) 105ºC, and (D) 121ºC for 30 min. Peaks: 1 = (all-*E*)-lutein; 2 = (all-*E*)-zeaxanthin; 3 = (13-*Z*)-lutein; 4 = (13'-*Z*)-lutein; 5 = (13-*Z*)-zeaxanthin; 6 = (9-*Z*)-lutein; 7 = (9'-*Z*)-lutein; and 8 = (9-*Z*)-zeaxanthin.

Table 4.9 Tentative identification and relative abundance for (all-*E*) and (*Z*)-forms of lutein found in degummed yellow silk cocoons from various heat treatments.

Temp. (ºC)	Time (min)	Relative abundance (%)												Tentative identified compound (%) [b]			
		Lutein					Zeaxanthin		Un-identified		(all-*E*) [a]	(13 or 13'-Z) [a]	(9 or 9'-Z) [a]	(all-*E*)	(13 or 13'-Z)	(9 or 9'-Z)	Ratio of *E/Z*
		(13-*Z*)	(13'-*Z*)	(all-*E*)	(9-*Z*)	(9'-*Z*)	(13-*Z*)	(all-*Z*)	*cis* [c]	unknown							
85	30	1.53^{f}	1.18^{g}	75.43^{ab}	0.74^{f}	-	0.43^{f}	12.60^{a}	4.95^{abc}	3.15^{a}	88.03^{a}	3.13^{f}	0.74^{g}	95.78^{a}	3.41^{g}	0.81^{g}	96:4
	60	1.74^{ef}	1.61^{gf}	78.04^{a}	0.86^{f}	-	0.56^{f}	12.34^{a}	2.14^{ef}	2.69^{ab}	90.38^{ab}	3.92^{fg}	0.86^{g}	94.97^{ab}	4.12^{fg}	0.91^{g}	95:5
	90	1.84^{ef}	1.42^{gf}	78.64^{a}	0.66^{f}	-	0.47^{f}	12.44^{a}	1.93^{f}	2.60^{ab}	91.08^{a}	3.73^{fg}	0.66^{g}	95.40^{a}	3.91^{fg}	0.69^{g}	95:5
	120	2.50^{def}	1.61^{gf}	78.05^{a}	0.66^{f}	-	0.60^{f}	12.41^{a}	2.10^{ef}	2.07^{bc}	90.45^{ab}	4.71^{g}	0.66^{g}	94.39^{ab}	4.92^{fg}	0.69^{g}	94:6
105	15	2.67^{def}	1.57^{gf}	77.69^{a}	1.47^{ef}	0.65^{e}	0.71^{f}	10.52^{bc}	6.32^{def}	-	88.21^{b}	4.95^{ef}	2.13^{fg}	92.57^{bc}	5.20^{ef}	2.23^{fg}	93:7
	30	3.10^{de}	2.21^{gef}	72.68^{bc}	1.86^{ef}	0.94^{e}	0.84^{f}	11.64^{ab}	5.32^{cd}	1.40^{cd}	84.32^{c}	6.15^{e}	2.80^{ef}	90.40^{c}	6.59^{e}	3.01^{ef}	90:10
	60	3.76^{d}	2.68^{def}	71.51^{cd}	2.47^{de}	1.58^{de}	1.09^{ef}	9.77^{c}	6.37^{abcd}	1.09^{cd}	81.28^{d}	7.53^{d}	4.05^{e}	87.53^{d}	8.11^{d}	4.36^{e}	88:12
	90	5.10^{c}	4.29^{b}	69.34^{d}	3.47^{cd}	2.48^{d}	1.56^{e}	7.78^{e}	7.06^{bcd}	-	77.12^{e}	10.95^{c}	5.96^{d}	82.02^{e}	11.65^{c}	6.34^{d}	82:18
	120	6.28^{bc}	2.82^{cdef}	65.77^{e}	4.26^{c}	3.65^{c}	1.76^{de}	9.40^{cd}	7.34^{de}	-	75.16^{e}	10.86^{c}	7.92^{c}	80.01^{e}	11.57^{c}	8.43^{c}	80:20
121	15	8.35^{a}	4.62^{b}	56.40^{f}	6.08^{b}	4.67^{b}	1.70^{de}	10.55^{bc}	9.47^{abc}	-	66.96^{f}	14.68^{b}	10.75^{b}	72.48^{f}	15.89^{b}	11.63^{b}	72:28
	30	6.63^{b}	6.12^{a}	55.49^{fg}	6.61^{ab}	5.56^{b}	2.38^{d}	9.80^{c}	7.40^{abc}	-	65.29^{f}	15.14^{b}	12.17^{b}	70.51^{g}	16.35^{b}	13.15^{b}	71:29
	60	6.06^{bc}	4.22^{bc}	52.90^{gh}	7.61^{a}	6.77^{a}	3.79^{c}	9.47^{cd}	8.60^{abc}	0.86^{d}	62.37^{g}	14.07^{b}	14.39^{a}	68.67^{gh}	15.49^{b}	15.84^{a}	69:31
	90	5.61^{bc}	3.61^{bcde}	53.02^{gh}	7.22^{ab}	7.45^{a}	5.44^{b}	8.41^{de}	9.25^{ab}	-	61.42^{g}	14.65^{b}	14.67^{a}	67.68^{hi}	16.15^{b}	16.17^{a}	68:32
	120	5.58^{bc}	3.89^{bcd}	51.23^{h}	7.12^{ab}	6.89^{a}	7.36^{a}	7.72^{e}	10.20^{a}	-	58.95^{h}	16.83^{a}	14.02^{a}	65.65^{i}	18.74^{a}	15.61^{a}	66:34

Note: [a] Relative abundance of total (all-*E*), (13-*Z*) and (13'-*Z*), and (9-*Z*) and (9'-*Z*)-isomers of lutein and zeaxanthin, separated by C_{30}-RP HPLC. [b] Values were calculated to total identified compound. [c] Relative abundance of unidentified (*Z*)-isomers of lutein. Different letters on the same column indicate significant differences ($p \leq 0.05$).

Table 4.10 Tentative identification and relative abundance for (all-*E*) and (*Z*)-forms of lutein found in degumming solution from various degumming processes of yellow silk cocoons.

Temp. (ºC)	Time (min)	Relative abundance (%)												Tentative identified compound (%) [b]			
		Lutein					Zeaxanthin		Un-identified		(all-*E*)[a]	(13 or 13'-Z)[a]	(9 or 9'-Z)[a]	(all-*E*)	(13 or 13'-*Z*)	(9 or 9'-*Z*)	Ratio of *E*/*Z*
		(13-*Z*)	(13'-*Z*)	(all-*E*)	(9-*Z*)	(9'-*Z*)	(13-*Z*)	(all-*E*)	*cis*[c]	unknown							
85	30	-	-	100.00^{a}	-	-	-	-	-	-	100.00^{a}	-	-	100.00^{a}	-	-	100:0
	60	-	-	100.00^{a}	-	-	-	-	-	-	100.00^{a}	-	-	100.00^{a}	-	-	100:0
	90	-	-	100.00^{a}	-	-	-	-	-	-	100.00^{a}	-	-	100.00^{a}	-	-	100:0
	120	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
105	15	2.63^{d}	1.63^{c}	76.73^{b}	2.54^{d}	1.22^{d}	-	12.02^{abc}	1.21^{c}	2.03^{a}	88.74^{b}	4.25^{e}	3.76^{d}	91.72^{b}	4.39^{e}	3.89^{d}	92:8
	30	2.47^{d}	1.64^{c}	75.63^{b}	2.15^{d}	1.18^{d}	0.55^{e}	12.15^{abc}	2.28^{bc}	1.94^{b}	87.78^{b}	4.66^{e}	3.34^{d}	91.66^{b}	4.86^{e}	3.48^{d}	92:8
	60	1.91^{d}	1.23^{c}	76.02^{b}	2.29^{d}	1.06^{d}	0.62^{e}	12.47^{ab}	2.66^{bc}	1.75^{c}	88.49^{b}	3.76^{e}	3.35^{d}	92.57^{b}	3.93^{e}	3.50^{d}	93:7
	90	1.82^{d}	1.36^{c}	76.83^{b}	2.04^{d}	0.76^{d}	0.66^{e}	13.85^{a}	1.62^{c}	1.05^{d}	90.68^{b}	3.85^{e}	2.81^{d}	93.16^{b}	3.95^{e}	2.88^{d}	93:7
	120	2.19^{d}	1.66^{c}	75.67^{b}	2.82^{d}	1.35^{d}	0.80^{de}	13.19^{a}	1.30^{c}	1.02^{e}	88.86^{b}	4.65^{e}	4.17^{d}	90.97^{b}	4.76^{e}	4.27^{d}	91:9
121	15	4.84^{c}	3.86^{a}	65.88^{c}	5.86^{c}	4.28^{c}	1.32^{de}	9.64^{bcd}	5.04^{abc}	-	75.52^{c}	10.02^{d}	10.14^{c}	78.93^{c}	10.47^{d}	10.60^{c}	79:21
	30	5.95^{bc}	4.32^{ab}	61.14^{c}	6.29^{bc}	5.10^{c}	1.65^{cd}	9.14^{d}	7.01^{ab}	-	70.28^{d}	11.92^{cd}	11.39^{c}	75.09^{d}	12.74^{c}	12.17^{c}	75:25
	60	5.93^{bc}	4.70^{ab}	54.48^{d}	8.28^{a}	8.44^{ab}	2.29^{bc}	8.35^{d}	8.55^{ab}	-	62.83^{e}	12.93^{bc}	16.72^{ab}	67.94^{e}	13.98^{bc}	18.08^{ab}	68:32
	90	6.76^{b}	4.38^{ab}	53.39^{d}	7.56^{ab}	7.54^{b}	2.95^{b}	9.31^{d}	9.25^{ab}	-	62.70^{e}	14.08^{b}	15.09^{b}	68.24^{e}	15.33^{b}	16.43^{b}	68:32
	120	7.93^{a}	4.83^{a}	49.78^{d}	9.17^{a}	9.65^{a}	4.96^{a}	7.24^{d}	9.03^{a}	-	57.03^{f}	17.72^{a}	18.82^{a}	60.94^{f}	18.94^{a}	20.11^{a}	61:39

Note: [a] Relative abundance of total (all-*E*), (13-*Z*) and (13'-*Z*), and (9-*Z*) and (9'-*Z*)-isomers of lutein and zeaxanthin, separated by C_{30}-RP HPLC. [b] Values were calculated to total identified compound. [c] Relative abundance of unidentified (*Z*)-isomers of lutein. Different letters on the same column indicate significant differences ($p \leq 0.05$).

Table 4.9 and 4.10 show the distribution of lutein isomers, represented as percent relative abundance by mean of peak area determination, in the degummed cocoons and pigmented-sericin solutions, respectively. The relative amount of (*Z*)-isomers of lutein and zeaxanthin increased with increasing temperature and time of degumming process. The predominant (13-*Z*)-isomers of lutein showed the highest proportion at 121ºC for 15 min ($p<0.01$) that, however, slightly decreased with longer period of time. These results indicated that increase in the treatment time at 121ºC resulted in the extent of thermal degradation corresponding with previous described by Socaciu (2007). Different results in each heating stage may point to the difference in the activation energies of the isomerization, or even the epoxidation and chain breaking degradation (Li and Han, 2008; Milanowska and Gruszecki, 2005; Socaciu, 2007). In this study, the accumulation of (13-*Z*)-isomers of lutein was almost found in the extracts from degumming products at 85 and 105ºC. At 121ºC, the accumulated (13-*Z*)-isomers decreased with increase in treatment time, meanwhile the predominant accumulation of (9-*Z*)-isomers was observed. These incidents may be described as the difference in the activation energies of the isomerization at the near-end position requiring much more activation energy than about the central double bond (Socaciu, 2007). In addition to lutein, the (13-*Z*)-isomers of zeaxanthin were identified without detectable (9-*Z*)-isomers or others. Similar results were observed between the heat-induced and light-induced isomerization of (all-*E*)-zeaxanthin that agreed with previous study of Milanowska and Gruszecki (2005). Moreover, with the worst heating condition, the *trans* to *cis* (*E/Z*) ratio of 66:34 of lutein was obtained. More than 90% of (all-*E*)-lutein and (all-*E*)-zeaxanthin remained in the degummed cocoons after degumming at 105ºC for 15-30 min or at 85ºC. Besides, at least 70% of both

xanthophylls still remained in the cocoons after degumming at 121ºC for 15-30 min. Comparing to degummed cocoons, the pigment compositions were likely stable against thermal treatment, since there was no significant difference among the relative amount of lutein, zeaxanthin and their geometric isomers after prolonged heating at 105ºC (p<0.05). A proximately 91-93% of (all-*E*)-configuration of lutein and zeaxanthin remained in the pigmented-sericin solutions, whereas 80-93% of which remained in the degummed cocoons. As a result of the degumming at 121ºC for 15-30 min, the composition of lutein in the degumming solutions was found predominantly as a native form of (all-*E*)-lutein (61-65%), while that of remaining lutein in the degummed cocoons was found in a lower amount (55-56%). Similarly to the *E/Z* ratio of xanthophylls, that the retention of (all-*E*)-xanthophylls in the degummed cocoons (71-72%) was lower than comprising in the pigmented-sericin solutions (75-79%). These results indicated that the stability of (all-*E*)-xanthophylls in the pigmented-sericin solutions probably attributed to their susceptibility as affected by surrounding medium (Lavecchia and Zuorro, 2006).

It is interesting to note that the higher proportion of lutein isomers (27-34%) was found in a part of degummed cocoons. In view of this point, the physiological factor was probably affected on the *E/Z* isomerization of (all-*E*)-lutein. The presence of lutein with sericin in the pigmented-sericin solution was different from free form of lutein found in the degummed cocoons. As indicated by the change of its solubility that was totally modified in aqueous medium. The heating process promotes the isomerization of (all-*E*)-lutein, its usual configuration, to the (*Z*)-form due to its susceptibly unsaturated structure. However, slight difference in the absorption spectra between lutein in a part of the pigmented-sericin solution and standard (all-*E*)-lutein

probably associated with the limited isomerization as an effect of protein barrier or electron transferring from the carotenoid-free radical adduct to ground state (Rodriguez-Amaya, 2001). Therefore, apart from utilizable lutein in a free form, presence in the sericin-lutein complexes likely increased the stability of (all-*E*)-lutein.

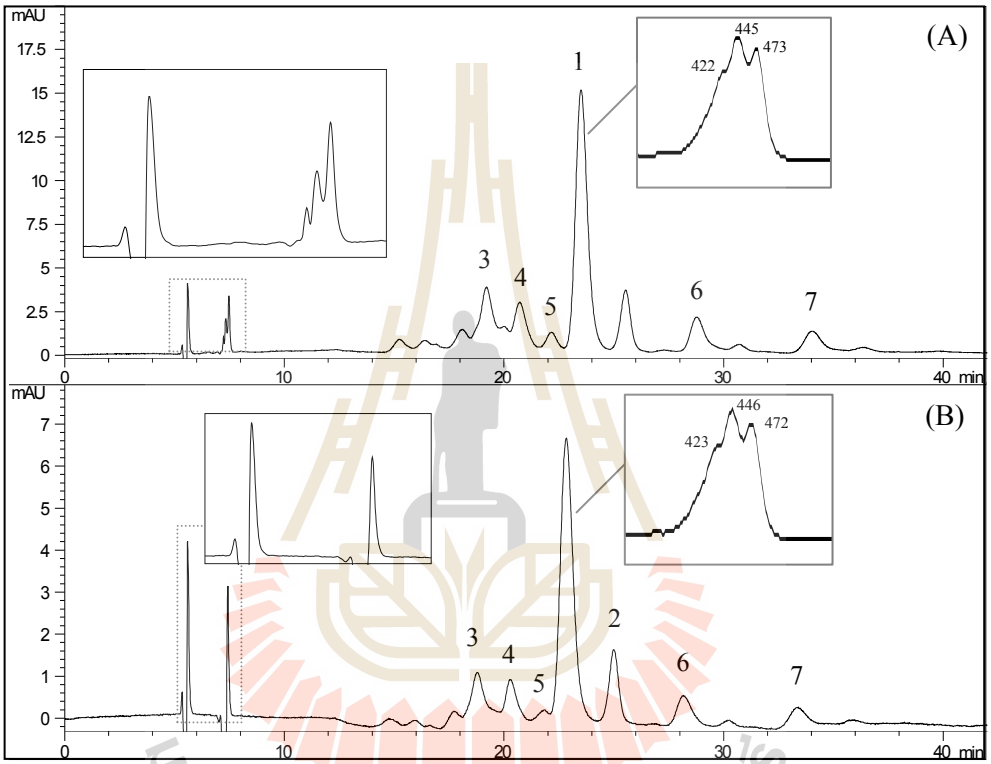

Figure 4.10 C_{30}-RP HPLC chromatograms of lutein extracts from (A) yellow silk cocoons, degummed at 121ºC for 15 min, and (B) degumming solution.

4.4 Stability study of lutein extract

For the stability test, the sample of (all-*E*)-lutein and its isomers was extracted from yellow silk cocoons after degumming at 121ºC for 15 min. By the use of this condition, similar patterns of the pigment distribution in both degummed cocoons and pigmented-sericin solution was obtained (Figure 4.10). The lutein extract contained

(all-*E*)-lutein, (all-*E*)-zeaxanthin, and their (*Z*)-geometric isomers with trace amount of unidentified (*Z*)-lutein that shown the *E/Z* ratio for 72:28 and 79:21 in the degummed cocoon and the pigmented-sericin solution, respectively. The pigmented-sericin solution has the L/S ratio of 3.26 g/kg of total protein content. The stability test was conducted in both lutein extract from the degummed cocoons and the pigmented-sericin solution.

4.4.1 Effect of temperature, light, and organic solvents on the stability of lutein

The stability of lutein from the degummed cocoons was studied in the crude extract, prepared with various solvent systems and dried, for 12 weeks at different temperature. As of the results shown in Figure 4.11, lutein contents dramatically decreased at 55˚C, but slightly changed at 4 and 25˚C. Only a 9.9% of the initial lutein content was lose after storage for 21 days at 4ºC. Light exposure (3,100-3,161 Lux) of dried crude extract caused the degradation of total lutein. The initial lutein content was remained (79.1%) for only the first 7 days at 25˚C. After an induction time of 21 days, the total volume of lutein decreased from 79.1% to 65.1% of the initial content, whereas, a 91.3-97.0% and 63.7% of the initial lutein content was remained within 21 and 84 days, respectively, without light at the same temperature. These results indicated that light and heat exposure largely affected on the lutein degradation accordingly with previous studies (Aman, Schieber and Carle, 2005; Chen, Peng and Chen, 1996; Lin and Chen, 2005; Tang and Chen, 2000).

Figure 4.12 shows the concentration changes of lutein in various solvent systems during storage periods of 12 weeks at 55ºC. Comparing to dried extract, thermal stability of lutein was enhanced in the solutions of organic solvents. More

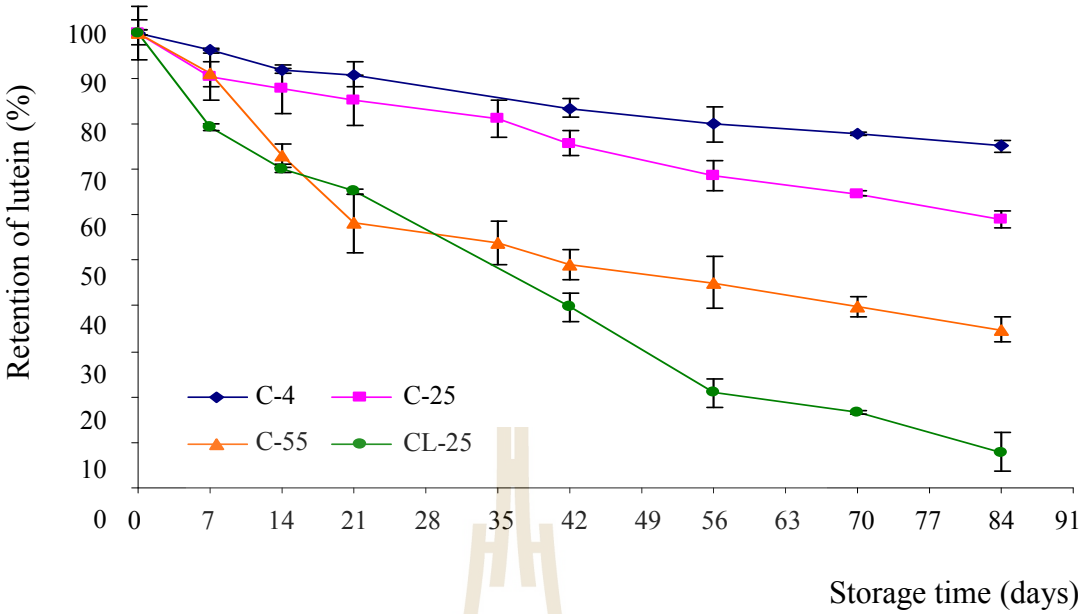

Figure 4.11 Stability plots: dried crude extract of lutein storied at various temperatures in the dark (C) and light (CL) conditions.

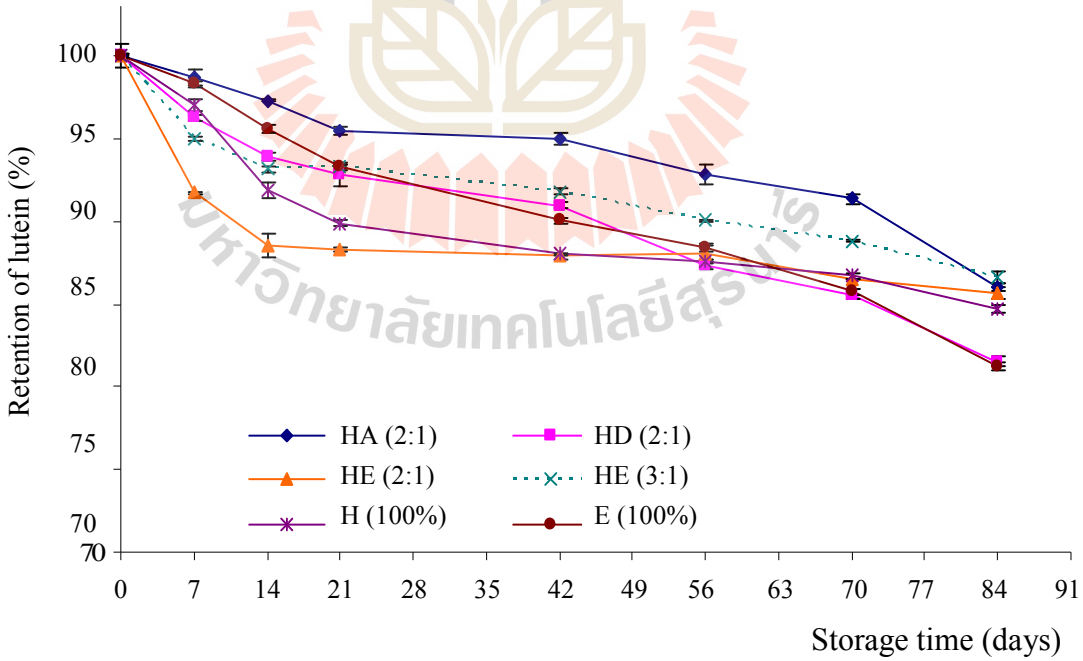

Figure 4.12 Stability plots: the extract solution of lutein in various solvents storied at 55ºC under dark.

than 81.2% of lutein still remained in the solutions until 12 weeks of the incubation. There was no significant change of lutein concentration when was stored in solvents.

The stability of lutein has been studied in various systems. Under UV light, the concentration of lutein and its esterified forms such as lutein monomyristate and lutein dimyristate decreased very quickly to the remaining amount of 42.3%, 78.2%, and 78.6%, respectively, after incubated at 10˚C for 3 days. (Subagio, Wakaki and Morita, 1999). For the conditions used in this study under fluorescent light for 14 days, the initial concentration of lutein reduced to 73.8%. The accumulation of light absorption energy during prolonged period was sufficiently responsible for activation of degradative oxidation that, consequently, reduced in lutein concentration. Meanwhile, the isomeric composition of lutein exhibited a time-dependent degradation following light exposure. The initial ratio of (*Z*)- to (all-*E*)-isomer (4.4:95.6) of standard lutein was increased slightly to 10.5:89.5 after light exposure at 3,750 Lux as demonstrated in previous study (Aman, Schieber and Carle, 2005). Likewise, in isolated chloroplasts, the (*Z*)- and (all-*E*)-lutein pattern was only slightly changed from the initial 12.6:87.4 ratio to 11.9:88.1 after illumination over 60 min. In the other hand, heat was known as another major cause of isomerization and, particularly, degradation. The effect of various thermal processing on the decrease in lutein content in vegetables was investigated extensively in a past decade (Aman, Schieber and Carle, 2005; Chen, Peng and Chen, 1996; Updike and Schwartz, 2003). Boiling at 98˚C for 60 min leaded to decrease in the initial concentration of standard lutein by 16.2%, while lutein in chloroplast isolated from spinach decreased by 28%. The amount of lutein also decreased with increasing storage temperature and time. Obviously, in green-leafy vegetables, lutein occurring as chlorophyll-lutein complex

was more susceptible to heat than in free form, since chlorophylls act as sensitizers of lutein degradation and isomerization. However, the stability of carotenoids including lutein in the vegetables was enhanced when occurred naturally in chloroplasts as the carotenoids-protein complexes (Aman, Schieber and Carle, 2005). This inverse effect was probably observed in unpurified lutein, because the physiochemical and biological characteristics associated with its thermal-induced or even light-induced isomerization and degradation. In this study, lutein content in dried extract mostly degraded during light exposure at 25ºC, whereas small changes under dark was found at the same temperature. Additionally, the degradation of lutein in the solvent mixture was slightly changed at 55˚C under dark as in contrast with dried lutein extract that lutein content was largely decreased after the earliest week. In view of this point, to keep the extract of lutein in a certain solvent system is a way to improve the stability of lutein. Moreover, these evidences suggest that lutein stability was not only varied on the external factors, such as heat and light, but its different natural existing form and sources were also considerable.

4.4.2 Thermal stability of lutein in sericin-lutein complex

The effect of storage temperature on the concentration changes of lutein in the pigmented-sericin solutions is illustrated in Figure 4.13. After 12-hours of incubation at 25, 55, 85 and 100ºC, the amount of lutein decreased from the initial concentration to 64.01, 76.09, 86.26 and 89.04% remaining, respectively. No significant change was observed for lutein incubated at studied temperature. It is astounding that the greater reduction of lutein in the pigmented-sericin solutions was found at the incubation temperature of 25 and 55ºC, whereas the incubation at higher temperature resulted in a slight change of lutein concentration. These results implied that the

presence of lutein in a form of sericin-lutein complexes contributed to the alteration of thermal susceptibility of lutein.

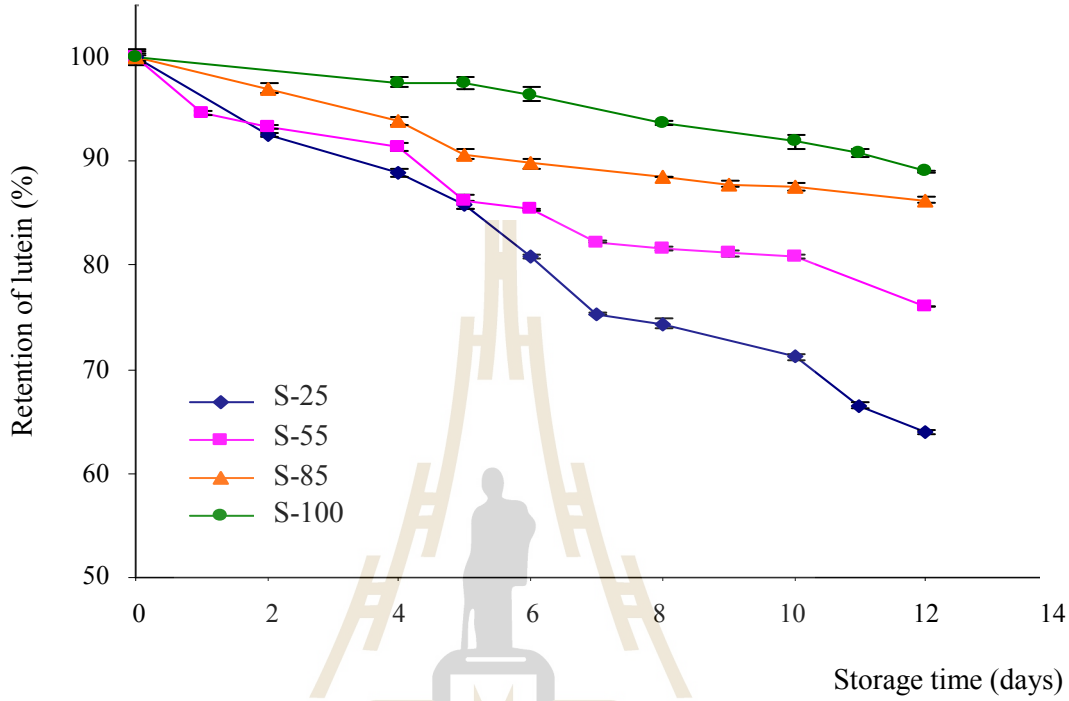

Figure 4.13 Stability plots: lutein in pigmented-sericin solutions storied at various temperatures under dark.

Lutein dissolved in degumming solution might possibly exist within the structure of silk protein sericin different from free lutein in degummed cocoons. As indicated by the change of its solubility that was totally modified in aqueous medium. Heating promotes isomerization of (all-*E*)-lutein, its usual configuration, to the (*Z*)-form due to its susceptibly unsaturated structure (Chen and Chen, 1993; Chen, Peng and Chen, 1996; Lin and Chen, 2005; Schieber and Carle, 2005). However, the results found in this study indicated that lutein existing as a sericin combination promoted its stability against heat. In view of this point, thermal-induced isomerization as well as

thermal degradation probably diminished by the effect of protein barrier or electron transferring from the carotenoid-free radical adduct to ground state (Rodriguez-Amaya, 2001). Therefore, it was remarkably that lutein comprising in pigmented sericin solution trended to be stable against heat than existing in its free form.

4.4.3 Degradation kinetics

The correlation coefficients for reaction kinetic models were calculated in order to determine the most correlated one (data not shown). The change of the lutein quantity due to heat and light during storage at various temperatures correlated to linear equation (R^2>0.93) and first-order reaction kinetic. The highest degradation rate of lutein in dried crude extract was 0.001 at 25˚C under light, and two folds higher than that stored at 55˚C. Compared to the study of Tang and Chen (2000) where destructive rate constants of lutein in freeze-dried carotenoid powder from carrot pulp waste for 0.004, 0.006, 0.009 and 0.013 were investigated during storage at 4, 25 and 45˚C under dark, and light at 25˚C, respectively. The rate constant of photo degradation, shown in Table 4.11, was 10.8 times lower, whereas 18.0 folds lower was found in thermal degradation. Whatever, carrot pulp waste possibly contained enzymes which may be dissolved during cell disruption. These should be noted that lutein powder from carrot pulp waste was more susceptible to heat and light than that from yellow silk cocoons, since not only different forms of lutein be found, but enzymatic reaction proceeding oxidized-chain reaction may also take place (Dutta, Chaudhuri and Chakraborty, 2005). Out of that point, the experimental data obtained in this study imply more complex first-order reaction kinetics that can be divided into two stages corresponding to recent studies of Bezbradica et al. (2005). Figure 4.13 illustrates the two-stage first order kinetic of lutein degradation in the crude extract

from yellow silk cocoons. The thermal degradation followed first-order kinetic during both stages, but the rate constants of degradation were larger in the first stage. However, by the effect of light, the lutein degradation in the second stage consequently occurs more rapidly.

Table 4.11 Two-stage first-order rate constant [a] and corresponding correlation coefficient.

Temp. (°C)	Storage condition	k x 10^{-4} (h^{-1})	R	k_1 x 10^{-4} (h^{-1})	R_1	k_2 x 10^{-4} (h^{-1})	R_2
4	dark/dried	-1.402	0.9777	-2.597	0.9947	-1.245	0.9781
25	dark/dried	-2.449	0.9855	-1.630	0.9938	-2.453	0.9879
55	dark/dried	-5.005	0.9268	-11.002	0.9716	-3.410	0.9921
25	light/dried	-11.921	0.9769	-8.386	0.9378	-13.652	0.9756
55	dark/HA (2:1)	-0.624	0.9202	-0.925	0.9900	-0.644	0.8171
	dark/HD (2:1)	-0.877	0.9658	-1.869	0.9830	-0.804	0.9625
	dark/HE (2:1)	-0.493	0.5588	-3.613	0.9436	-0.183	0.8282
	dark/HE (3:1)	-0.553	0.8769	-2.107	0.9342	-0.434	0.9600
	dark/H (100%)	-0.700	0.8198	-2.229	0.9730	-0.364	0.9558
	dark/E (100%)	-0.944	0.9793	-1.411	0.9913	-0.871	0.9524

Note: [a] Average of triplicate determinations ± standard deviation. Values of k and R without subscribed, represent the rate constant and corresponding correlation coefficient of the stability data, and in otherwise, with subscribed 1 and 2 represent those of the first-stage and second-stage first-order kinetics, respectively.

First-order kinetics of carotenoid degradation has been reported on several experiments performed in low-moisture, solvent-based model systems, aqueous, and oil systems (Aman, Schieber and Carle, 2005; Bezbradica et al., 2005; Henry, Catignani and Schwartz, 1998; Lin and Chen, 2005; Minguez-Mosquera and Gandul-Rojas, 1994; Tang and Chen, 2000). Furthermore, two-stage first-order kinetics was

also observed by Desobry, Netto and Labuza (1997), and Bezbradica et al. (2005). In this study, the degradation of lutein followed first-order kinetics considerably and, however, fitted well with two-stage first-order kinetics. The rate constants for the degradation of lutein are listed in Table 4.11. Nevertheless, light exposure during storage period increased the degradation rate of the second stage closer to the first rate. Instead of two-stage first-order kinetic model (R^2 = 0.94-0.98), the lutein degradation by the light (3,100-3,161 Lux) during 12 weeks storage period fitted well with simple first-order kinetic model (R^2 = 0.98). This result corresponded to the study of Aman et al. (2005), where pure lutein solution (without food matrix) was more susceptible to light (3,750 Lux) at 20˚C for 60 min than boiling temperature at 98˚C. Furthermore, both photo-induced and thermal isomerization also occurred during storage, although photo-induced took place to some extent than another one. However, they found that isomeric compositions were not significantly changed. It may be due to low light intensity and short exposure period used in this study, resulting of marginal increase in (*Z*)-isomers of lutein. Besides, the central (*Z*)-isomers such as (13-*Z*)-lutein predominantly formed during heating or dark storage, required low activation energy for isomerization (Tang and Chen, 2000). These indicated that the degradation of lutein during heat and light exposure be, therefore, due to its loss in color after attacked on radicals or oxidizing species, known as bleaching process which involved interruption of the conjugated double bond system either by cleavage or by adduction to one of the double bonds (Krinsky and Yeum, 2003).

In other case of low temperature (4˚C), shown in Table 4.11, the degradation of lutein revealed lower correlation than others. This may cause by the minimal

change in the initial lutein content was observed during 12 weeks of studied period. In other words, carotenoids including lutein may participate in the propagation step of the oxidation process as chain-breaking antioxidants that scavenge reactive peroxyl radicals (Jiménez-Escrig et al., 2000). At low activation energy from outside, the energy of existing reactive peroxyl radicals was transferred to the carotenoid molecule to ground state or low-energy free radicals and peroxyl radical-carotenoids adduct which then, eventually, may return to ground state through allylic hydrogen abstraction or energy dissipation by interaction with surrounding solvents (Dutta, Chaudhuri and Chakraborty, 2005; Krinsky and Yeum, 2003; Martin et al., 1999; Stahl and Sies, 2003). These may explain why the stability of lutein in both dried extract kept at 4˚C and hexane-ethanol solution kept at 55˚C under dark were enhanced. Up to now, mechanism of the degradation was not clear. However, evidences suggest that auto-oxidation be occurred at the first stage of destruction caused by peroxides or high energy radicals accumulating from the first step of the extraction (Bezbradica et al., 2005), and then activating chain reaction in auto-oxidation in the early period of the storage with the initial rate 2.57 μM min^{-1} for lutein destruction as reported by Woodall et al. (1997). Whereas, in slower stage, the free radical quenched by lutein in the first step causing low energy radicals, and then lutein reacted with this radical again. Finally, the degradation reaction was slowed down. When compared to the degradation rate of lutein in the hexane-ethanol solution to dried extract at 55˚C, the differences between rate constants in different conditions were observed in the second stage of the storage. Moreover, in the dried extract, the second rate of destruction was nearly to the rate of one-stage destruction (indicated as dot line in Figure 4.14C). In contrast, the degradation rate of lutein in the solution was

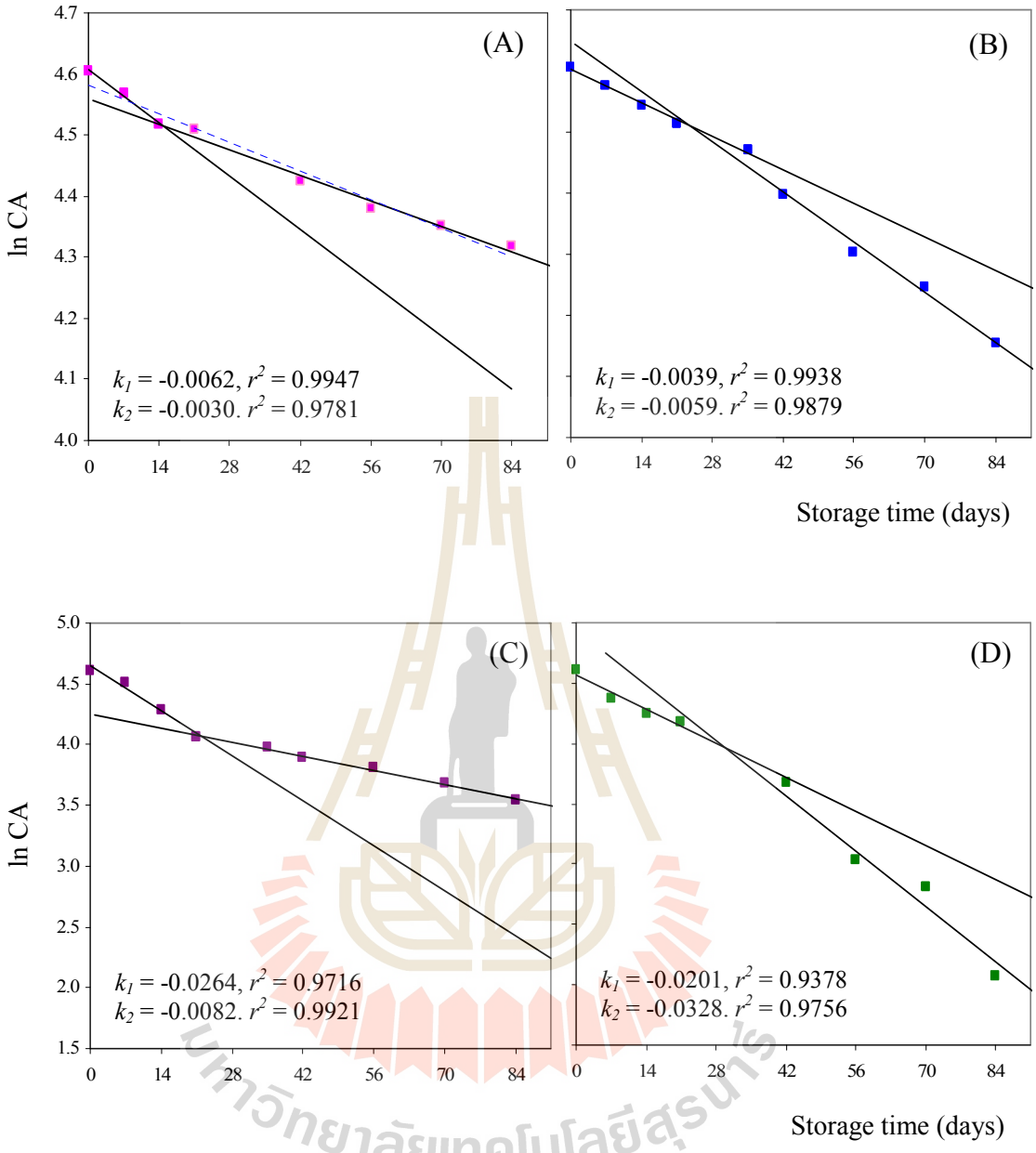

Figure 4.14 Two stage first-order kinetic model of the lutein degradation: (A) dried extract stored at 25˚C with and (B) without illumination, (C) 55˚C in the dark, and (D) 55˚C in hexane/ethanol (2:1, v/v).

obviously retarded (Figure 4.14D). These results suggest that the degradation of lutein in the two stages differ not only the rates, but also the mechanism, corresponding to previous study by Bezbradica's group (2005). The degradation of lutein as well as other carotenoids may only relate to auto-oxidation in the first stage, while during the second stage it properly occurs in reactions, independent to auto-oxidation. These indicated that the influence of the heat, light, and preparation method on the stability of lutein in the extract be the factors considered for the extraction.

Table 4.12 Degradation kinetic parameters and the half-life of lutein [a].

Storage time (weeks)	Temp. (°C)	Storage condition	k x 10^{-4}(h^{-1})	R^2	Half life ($t_{1/2}$) (months)	Ea (kJ mol^{-1})	R^2
Lutein extract from degummed cocoons							
6	4	dark/dried	-1.747	0.9748	5.5	21.338	0.9310
	25	dark/dried	-2.364	0.9325	4.1		
	55	dark/dried	-7.214	0.9293	1.3		
12	4	dark/dried	-1.402	0.9777	6.9	18.881	0.9997
	25	dark/dried	-2.449	0.9891	3.9		
	55	dark/dried	-5.005	0.9268	1.9		
12	25	light/dried	-11.921	0.9769	0.8	-	-
12	55	dark/HA (2:1)	-0.624	0.9202	15.4	-	-
		dark/HD (2:1)	-0.877	0.9658	11.0		
		dark/HE (2:1)	-0.493	0.5588	19.5		
		dark/HE (3:1)	-0.553	0.8769	17.4		
		dark/H (100%)	-0.700	0.8198	13.8		
		dark/E (100%)	-0.944	0.9793	10.2		
Lutein in pigmented-sericin solution							
12 (hours)	25	dark	-372.314	0.9842	0.03	16.392	0.9990
	55	dark	-211.751	0.9607	0.05		
	85	dark	-125.185	0.9301	0.08		
	100	dark	-98.565	0.9552	0.10		

Note: [a] Average of triplicate determinations ± standard deviation.

To quantify the storage stability of lutein extracted from yellow silk cocoons, its apparent half-life, the time required for the initial amount of lutein to disappear 50%, was determined. Additionally, the temperature dependence of k and apparent activation energy (Ea) were estimated by Arrhenius equation. The degradation kinetic parameters and the half-life of lutein were evaluated during 6 and 12 weeks of storage periods. Table 4.12 shows, half-life of lutein in the dried extract was approximately 4 months at room temperature (25˚C), and increased to around 7 months at 4˚C. In the other hand, the half-life was minimized to 2 months in the extracts kept at 55˚C. The lutein degradation increased with temperature. The degradation rate constant showed highest at 55˚C, and lowest at 4˚C. According to the apparent activation energies, the first-order kinetic of thermal degradation resulted in the activation energy of 18.88 kJ mol^{-1}. For lutein in the pigmented-sericin solution, an inverse effect of temperature on the degradation of lutein was observed. The degradation rate constant of lutein decreased with increasing in the incubation temperature similarly to its half-life that showed the highest degradation rate at 100ºC. These results imply the effect of surrounding medium on the modification of lutein stability. Despite, the kinetic studies on the degradation of lutein are intensively beneficial for developing both pharmaceutical and nutraceutical products of lutein, related papers have been rarely published. The comparison of obtained values of the activation energy to literature values, therefore, can not be achieved directly. The obtained activation energy, however, is lower than the value of 60.9 and 44.9 kJ mol^{-1} found in the degradation of lutein extracted from marigold flowers in sunflower and rice bran oils, respectively, at 25 and 40˚C for 10 days studied period (Lavacchia and Zuorro, 2006). Difference in these values probably attributed to the effect of the oil mediums. Besides, activation

energy of the oxidative degradation of lutein as well as lycopene and β-carotene in sunflower seed oil, heated individually at 75, 85 and 95˚C for 5, 12 and 24 hours, were 24.9, 19.8 and 26.2 kJ mol^{-1}, respectively, close to the value of lutein in this study (Henry, Catignani and Schwartz, 1998). Though, the results could not be directly compared with the literature values, since the experiments were conducted in different mediums which had different effect on the lutein stability depending on the composition existing (Aman, Schieber and Carle, 2005; Bezbradica et al., 2005). The activation energies of thermal degradation of lutein obtained in this study were found closely to the values obtained from those heating condition with solvent effect. Thus, it should be noted that the lutein extracted from yellow silk cocoons was susceptible to thermal degradation, likely to lutein and lutein esters from plant sources where both thermal and oxidative degradation were took places.

4.5 Antioxidant activities of lutein extract

4.5.1 The DPPH˙ free radical scavenging activity

Stable free radical DPPH˙ is often used for the evaluation of general radical scavenging capabilities of various antioxidants. DPPH˙ is a paramagnetic compound with an odd electron showing strong absorption band at 515-518 nm in methanol or ethanol-based solution (Chandrasekar et al., 2006; Milardović, Ivekovic and Grabaric, 2006; Sánchez, Larrauri and Saura-Calixto, 1998; Szabo et al., 2007; Thaipong et al., 2006). However, the antioxidant activity, represented as the DPPH˙ radical scavenging ability, was determined by a new measure of decrease in absorbance at 580 nm in this study, in order to avoid the interference of carotenoids and other compounds with a chromophore system (Jiménez-Escrig et al., 2000). In this study,

the DPPH· radical was reduced to form the stable non-radical DPPH-H with spontaneous change of the violet color to pale yellow by different concentrations of lutein, α-tocopherol, and BHT.

Table 4.13 Amount necessary to deplete the initial DPPH· by 50% (EC_{50}), time to reach the steady state at EC_{50} (T_{EC50}) and antiradical efficiency (AE) of lutein extract comparing with some antioxidant [a].

Antioxidant	EC_{50} (mol antioxidant mol^{-1} DPPH)	T_{EC50} (min)	AE ($1/EC_{50}T_{EC50}$)
Lutein extract	3.62 ± 0.02	2.50 ± 0.11	0.111
α-Tocopherol	1045.69 ± 0.12	7.95 ± 1.12	0.008
BHT	633.93 ± 8.62	6.32 ± 0.44	0.010

Note: [a] Average of triplicate determinations ± standard deviation.

The antioxidant activity of lutein extracted from yellow silk cocoons expressed in the amount of antioxidant able to reduce the initial DPPH· concentration to 50% (EC_{50}), the time taken to reach the steady state at EC_{50} (T_{EC50}), and the antiradical efficiency (AE) are shown in Table 4.13. The EC_{50} of lutein in 2.5% THF ethanolic solution interpolating from the regression model of dose-response curve was 3.62 mol lutein mol^{-1} DPPH·, and T_{EC50} was 2.50 min. The antiradical efficiency of lutein was higher than both α-tocopherol and BHT approximately 14 and 11 folds, respectively. These results correspond with the study of Jiménez-Escrig et al. (2000) where EC_{50} of lutein standard was 3.26 mol mol^{-1} DPPH·. However, the value of T_{EC50} (28.72 min) and AE (0.011) were higher than that found in this study. This may due to different condition of the different assay. In this study, the DPPH· assay was

performed at 37°C as biological condition, whereas another assay was done at room temperature. The EC_{50} values of α-tocopherol and BHT were also reported in different units. By converting in the unit of mmol L^{-1}, the obtained values were 0.23 and 2.62 (Zhang et al., 2006), 0.20 and 0.31 (Chaiyasit, McClements and Decker, 2005), 0.05 and 0.09 (Kim et al., 2005), 0.09 and 0.02 (Ancos, Gonzalez and Cano, 2000) for α-tocopherol and BHT, respectively. In addition, the values of T_{EC50} of both antioxidants were reported as 9.52, 22.12 min, and AE were 0.53, 0.09 (Ancos, Gonzalez and Cano, 2000), and 0.01, 0.01 (Zhang et al., 2006), respectively. These results indicate that the $DPPH^{\cdot}$ radical decreased in the order of lutein, α-tocopherol and BHT, corresponding to the results found in this study (Ak and Gulcin, 2008).

The main interest in the reactions of carotenoids with free radicals is whether carotenoids can give effective protection against diseases caused by oxidative stress (Polykov et al., 2001). The values of EC_{50}, T_{EC50} as well as antiradical efficiency (AE) are very potentially benefit for the formulation and development of both pharmaceutical and nutracuatical products with high effectiveness and safety. The EC_{50} value indicates the dose of antioxidant required for protection of cell against oxidative damage which related to preventive effect of certain diseases, and T_{EC50} represents the efficiency or reaction time of the antioxidants to react over free radicals. Also, AE is a new parameter used for comparison of interested antioxidants. Mechanisms of free radical scavenging have been rarely investigated. However, Miller et al. (1996) have explained that the relative abilities of carotenes and xanthophylls to scavenge the radical cation are influenced by the presence of functional groups with increasing polarities, as carbonyl and hydroxyl groups, in the terminal rings, as well as by the number of conjugated double bonds. Additionally,

their scavenging effects relate particularly to their ability to donate electrons or hydrogen atoms, and their relative behaviors to undergo oxidation. These correspond to previously reports where the structure-activity relationship in radical-scavenging reactions of some carotenoids and phenolic compounds was described (Bhm et al., 1997; Jiménez-Escrig et al., 2000; Mortensen and Skibsted, 1997; Sánchez-Moreno, Larrauri and Saura-Calixto, 1998; Woodall et al., 1997).

4.5.2 Ferric reducing antioxidant power assay

An automated test measuring the ferric reducing ability, the FRAP assay, is simple, rapid, and highly reproducible. At low pH (optimum pH 6.3) a ferroin analog, the Fe^{3+} complex of tripyridyltriazine $Fe(TPTZ)^{3+}$, is reduced by antioxidants to its intense blue colored form, Fe^{2+} complex $Fe(TPTZ)^{2+}$ which has maximum absorbance at 593 nm (Santas et al., 2008). The amount of Fe^{2+} complex $Fe(TPTZ)^{2+}$ in 4-min reaction was used for comparing the total reducing or antioxidant power among antioxidants. Results obtained as absorbance increase at 593 nm were expressed as mM Fe^{2+} equivalents L^{-1} (Figure 4.15). Various concentrations of lutein extracted from yellow silk cocoons in the ethanolic solution of 2.5% THF were used for the measurement and likewise in α-tocopherol and BHT for comparison. The reaction of α-tocopherol and BHT were rapidly reached the completion under 1 min, whereas lutein was under 2 min. When compared at the same concentration, the ferric reducing ability of lutein, however, was greater than others. The FRAP reaction kinetic of 1 mol L^{-1} lutein solution represented the activity nearly to that of 100 mol L^{-1} α-tocopherol and BHT, individually. These results reveal that the ferric reducing activity of lutein extract was higher than those of well-known antioxidants, generally used in food and cosmetic products like α-tocopherol and BHT. The FRAP value of

100 μmol L^{-1} extracted lutein was 47.2 mM L^{-1}. When compared to α-tocopherol (15.0 mM L^{-1}), lutein extract had higher reducing power (500.9 mM L^{-1}) for approximately 30 folds at a concentration of 300 μmolL^{-1} (p<0.05). The reducing power of BHT showed the lowest. At the same concentration of 3 mmolL^{-1}, BHT (18 mM L^{-1}) was 3.8 fold lower than α-tocopherol (69 mM L^{-1}). These results agree with the DPPH$^{\cdot}$ radical scavenging activity test.

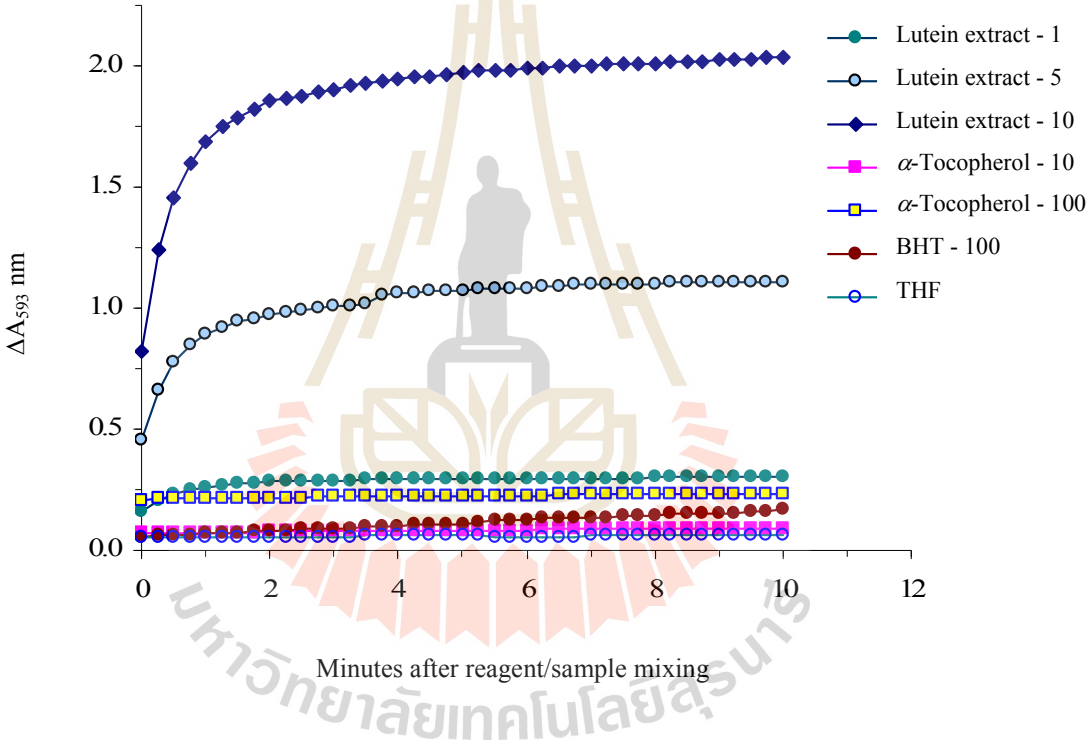

Figure 4.15 FRAP reaction kinetics with individual antioxidants: rate of increase in absorbance at 593 nm for 1, 5 and 10 μmol L^{-1} of lutein extract, and 10 μmol L^{-1} of α-tocopherol and BHT.

Instead of the antioxidant activity, the FRAP assay provides a very useful total antioxidant concentration without measurement and summation of the concentration of all antioxidants involved (Antolovich et al., 2002). The reduction

reaction of ferric-tripyridyltriazine $Fe(TPTZ)^{3+}$ complex is nonspecific, and any half-reaction which has a less-positive redox potential under the reaction conditions than the Fe^{3+}-Fe^{2+}(TPTZ) half-reaction will drive $Fe(TPTZ)^{3+}$ reduction (Benzie and Strain, 1996). Test condition favor reduction of the complex (straw colored) and, consequently, turn blue when a reductant as an antioxidant is present. An excess Fe^{3+} was used in the assay, and the rate limiting factor of Fe^{2+}(TPTZ), and hence color formation, is the reduction ability of the sample. The method was originally applied to plasma (Benzie and Strain, 1996) to other biological fluids, foods, plant extracts, etc (Jang et al., 2009; McInerney et al., 2007; Santas et al., 2008; Thaipong et al., 2006; Veneria et al., 2008).

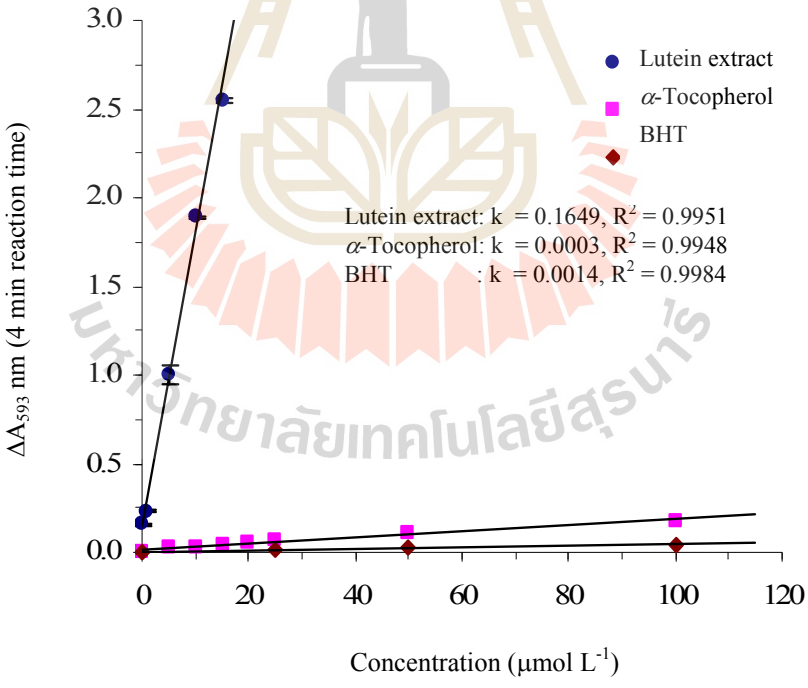

Figure 4.16 Linearity of FRAP: dose-response line for solution of lutein extract comparing with natural (α-tocopherol) and synthetic antioxidant (BHT).

The FRAP values obtained in this study could not be compared with the literature values directly, since the values were determined by means of total reducing ability of all antioxidants existing in the extracts. The antioxidant powers of a synthetic compound, octadeca-9,12-dienyl-3,4,5-hydroxybenzoate (GA-LA), garlic acid and α-tocopherol, studied by Jang et al. (2009), were 0.7, 30.2 and 2.3 mM kg^{-1} at a concentration of 50 μg mL^{-1}, respectively, whereas 15.0 mM L^{-1} of α-tocopherol at a concentration of 130 μg mL^{-1} (300 μmol L^{-1}) was found in this study. In addition, a tomato cultivar containing 1.08, 1.28 and 0.18 mg 100 g^{-1} of lycopene, β-carotene, and lutein had 4.1 mM kg^{-1} total antioxidant powers. Moreover, the antioxidant power increases accordingly with the concentration of antioxidants. Thus, the lutein extract from yellow silk cocoons was found as a predominant and most abundance antioxidant type. Hence, its antioxidant activity was found to be better free radical scavenger from other most likely antioxidants exited in the silk cocoons.

The dose-response characteristic of each antioxidant is illustrated in Figure 4.16. The results was obvious that the Fe^{2+} complex $Fe(TPTZ)^{2+}$ increased when the concentration of lutein increased. This showed that the kinetic reaction of the ferric reducing ability was first-order and dependent on the concentration. Whereas, the dose response of α-tocopherol and BHT was not likely increased when their concentrations increased. Thus, the kinetic reaction of these antioxidants was zero-order. Interestingly, since these results show the different mechanisms for the different antioxidants. Both α-tocopherol and BHT have the quenching or scavenging activities against the radical species with the mechanism of concentration independent, that their activity is not increased even their concentration in the reaction mediums are increased. While, lutein showed the first-order kinetic reaction, its

preventive ability to protection of the biological cells against oxidative damage is enhanced by increasing its concentration in the reaction medium. This information is essentially benefited for design of both pharmaceutical and neutraceutical products which achieve high efficiency effect, especially, without any undesired side effects.

4.6 Isolation and characterization of sericin-lutein complex

4.6.1 Molecular weight estimation of sericin

The sericin protein present in yellow silk cocoons showed good solubility in deionized distilled water as well as in all buffer systems used in this study. However, heat treatment in the degumming process was needed for improvement of sericin removal from the cocoon shells via thermal hydrolysis. The degumming solutions, obtained from various treatment times at 85, 105 and 121ºC, were analyzed for the molecular weight of protein compositions by SDS-PAGE. The molecular weight of the protein bands was determined with respect to molecular weight marker run in the same gel. Electrophoretic analysis of the crude pigmented-sericin in 12.5% gel shows differences in the protein profiles (Figure 4.17). Obtained crude sericin from degumming process at 85ºC showed six polypeptide bands with two dense bands around 250 and 105 kDa (Figure 4.17A). Similar protein profile of the degumming solutions was observed among several heating duration. At this degumming temperature, only a 0.0002-0.0006 mg of sericin was removed into a 100 mL of the degumming solutions. In contrast to higher temperature, sericin was almost separated from the cocoon shells that a 0.027-0.116 mg of sericin to 100 mL of the degumming solutions was obtained. The presence of a single smear band of sericin was observed for the pigmented-sericin solutions from heat treatment at 105 and 121ºC (Figure

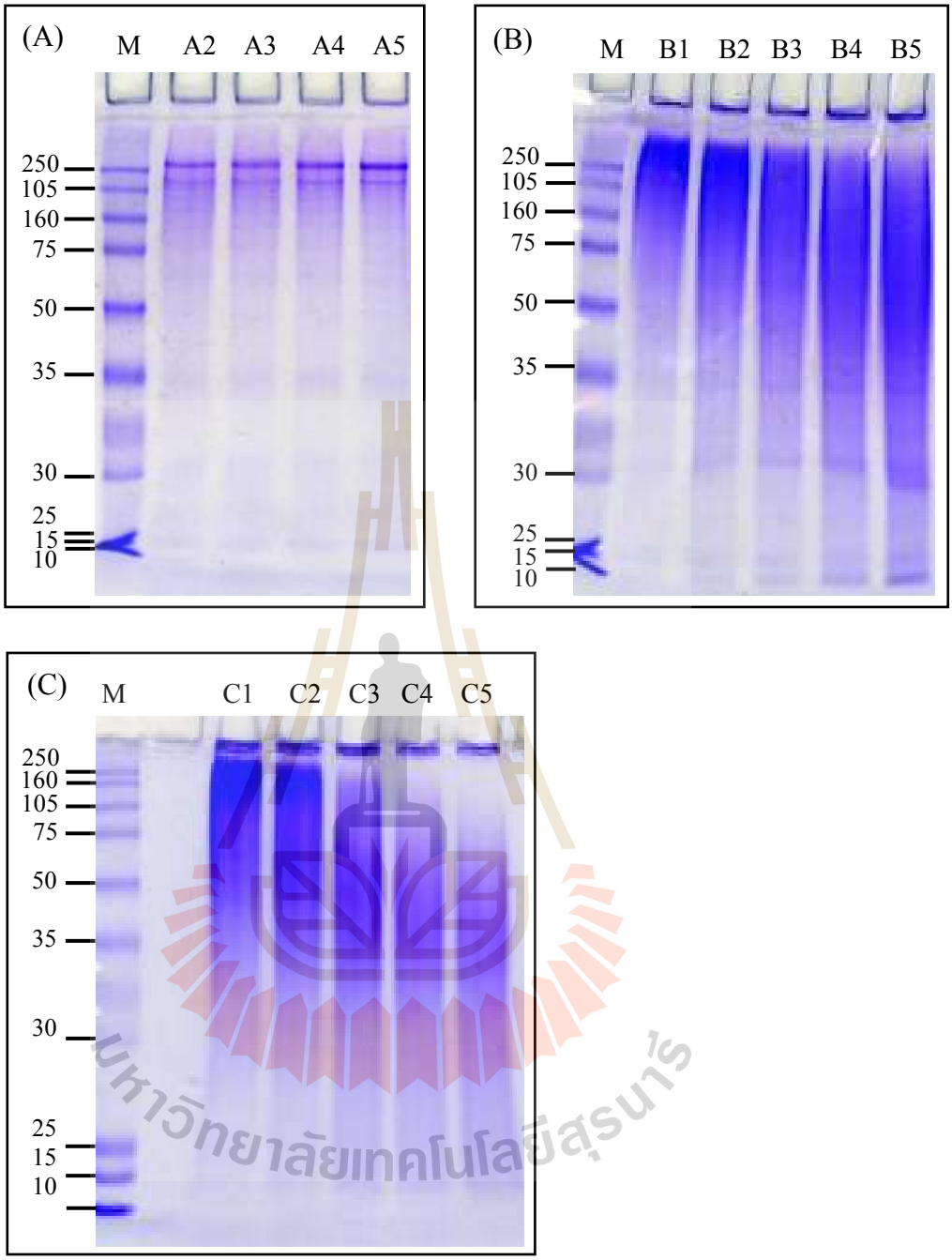

Figure 4.17 12.5% SDS-PAGE gel of sericin obtained from degumming process at 121ºC for 15 (C1), 30 (C2), 60 (C3), 90 (C4), and 120 (C5) min stained with Coomassie Brilliant Blue; left lane (M), molecular weight marker.

4.17B and 4.17C). It should be noted that increase in the heating period of time from 15 to 120 min at both temperature resulted in the lower molecular weight of principle protein combination. Because of degumming at 105ºC for 15 min, high molecular weight of sericin was determined to be greater than 160 kDa after approximately incubation time at 120 min caused the reduction in molecular weight to around 105-35 kDa. Likewise, the sericin components with the molecular weight higher than 50 kDa trended to be hydrolyzed into predominantly around 35-50 kDa after increase in the treatment time from 15 to 120 min at 121ºC. These different molecular mass of sericin may affect on its physicochemical and biological properties. Thus, to utilize the pigmented-sericin in either functional food or pharmaceutical products, further study was needed for evaluating chemical and functional properties of the isolated sericin and sericin-lutein complexes.

4.6.2 Isolation of lutein-binding protein

Cocoons of silkworm *B. mori* are found with a wide range of colors, yellow, orange, and red, that resulted from the proportions of lutein, β-carotene, α-carotene, and other xanthophylls in the cocoons (Tabunoki et al., 2004). However previous studies proved that lutein is the most important determinant of cocoon color of silkworm, *B. mori* (Tabunoki et al., 2002; Tabunoki et al., 2004) corresponding to the results found in this study. Apart from lutein extract, pigmented-sericin solution comprising lutein as sericin-lutein complex was also obtained from the pigment extraction process. As indicated by previous results, lutein in the sericin-lutein complex showed the modification of thermal degradation. Moreover, the absorption spectra of lutein extracted from this solution were very close to that of standard (all-*E*)-lutein, whereas the slight variation of the maximum absorption was found in

the extract from degummed cocoons. These results revealed the effect of protein barrier on the improvement of lutein stability. To characterize whether lutein existing in a protein-binding form or not, the pigmented-sericin solutions from various treatment times at 121ºC were used.

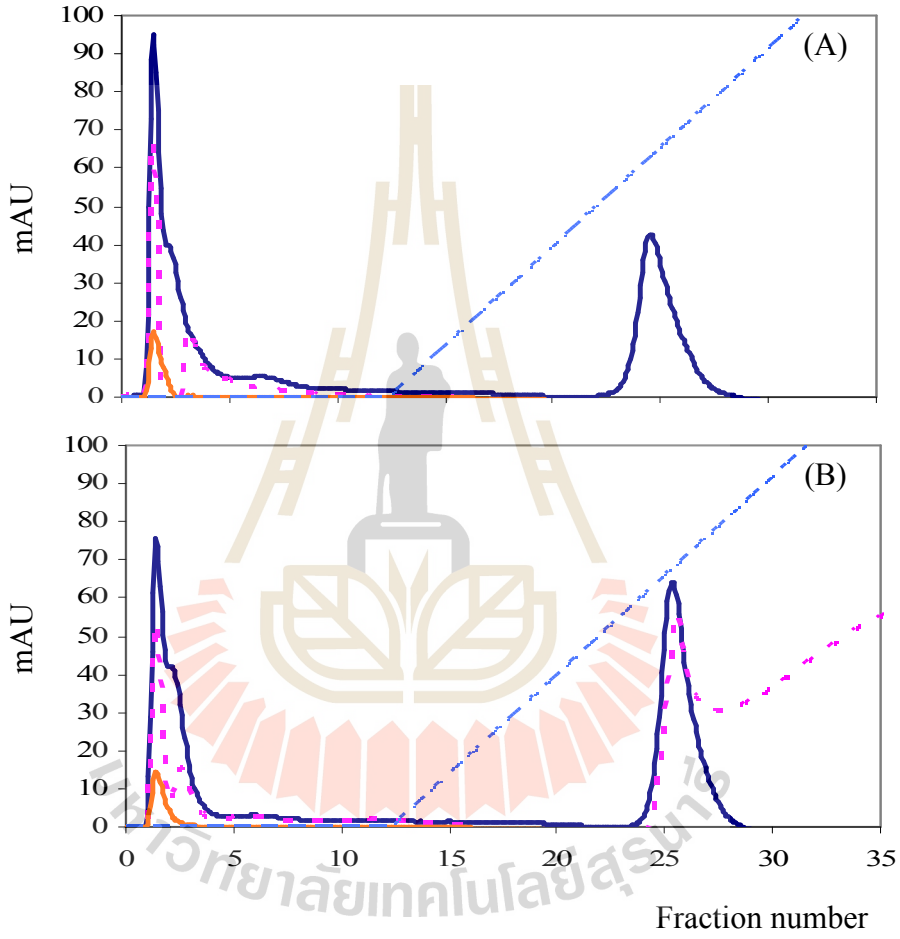

Figure 4.18 Elution profile of the lutein-binding protein on a DEAE column. The protein obtained from degumming process at 121ºC for 15 (A), 30 (B), 60 (C), 90 (D), and 120 (E) min were loaded onto a DEAE and eluted in a 1 M NaCl gradient (- - -) in 10 mM BisTris-HCl, pH 7.0. Fractions were monitored for protein at 280 nm (——), carotenoids at 460 nm (——), and peptides (– – –).

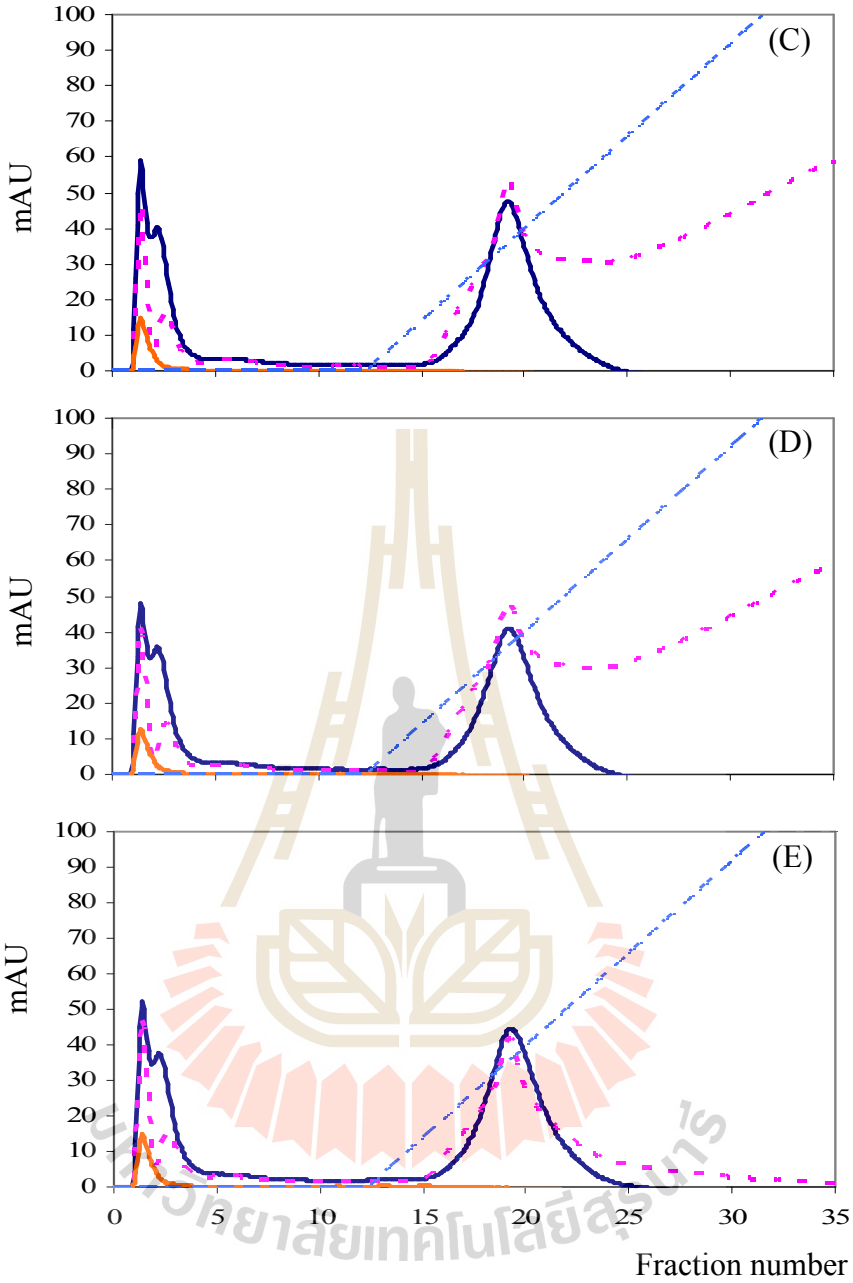

Figure 4.18 Elution profile of the lutein-binding protein on a DEAE column. The protein obtained from degumming process at 121ºC for 15 (A), 30 (B), 60 (C), 90 (D), and 120 (E) min were loaded onto a DEAE and eluted in a 1 M NaCl gradient (- - -) in 10 mM BisTris-HCl, pH 7.0. Fractions were monitored for protein at 280 nm (—), carotenoids at 460 nm (—), and peptides (– –). (Continued)

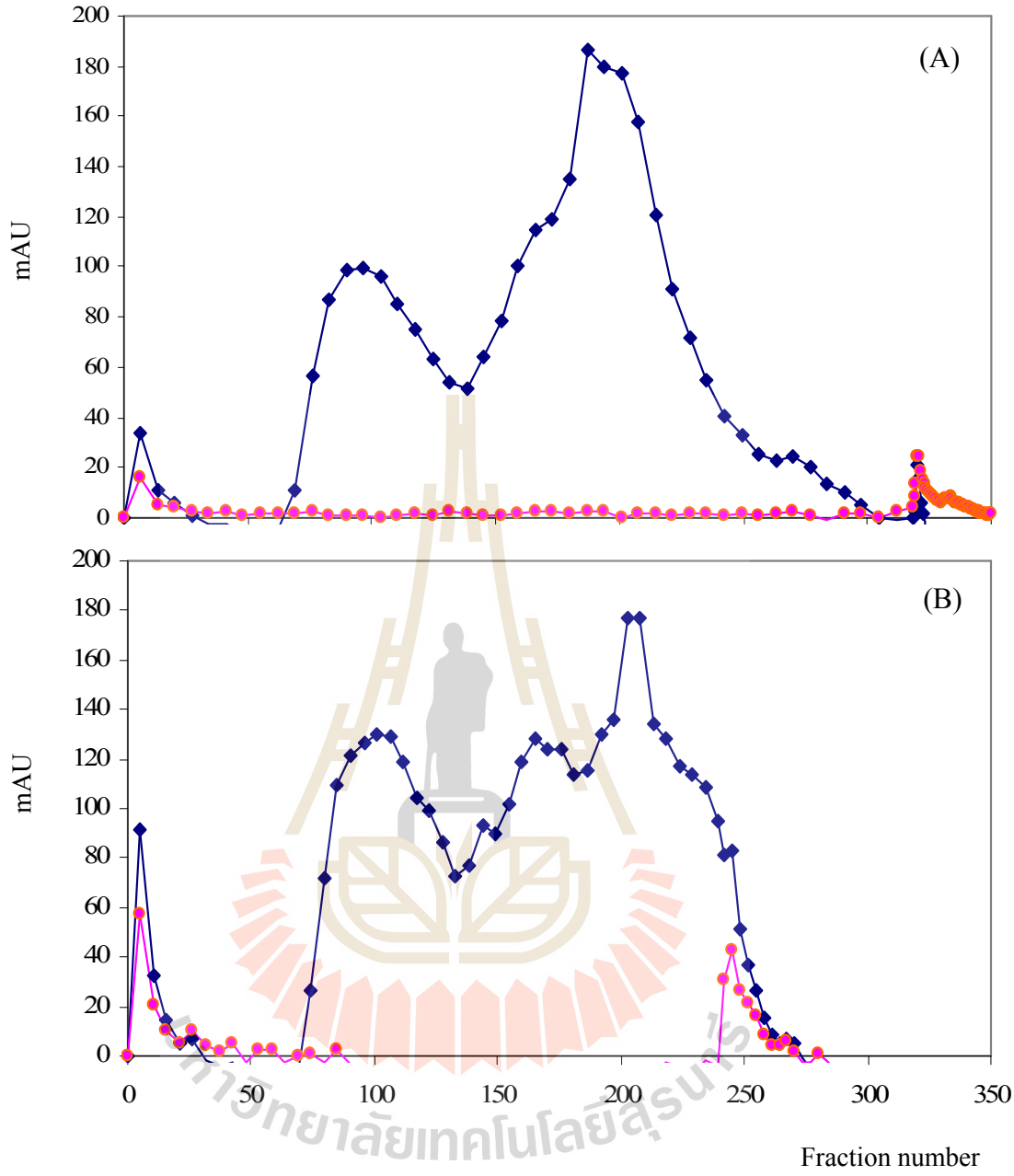

Figure 4.19 Elution profile of the lutein-binding protein on gel filtration. The pigmented-sericin solution obtained from degumming process at 121ºC for 15 (A) and 30 (B) min were loaded onto a Sephacryl S-200 column and eluted with 20 mM Tris-HCl buffer, pH 7.0. Fractions were monitored for protein at 280 nm (—◆—), carotenoids at 460 nm (—●—).

The elution pattern of the lutein-binding protein on a DEAE column was illustrated in Figure 4.18. The protein obtained from degumming process at all treatment time (15-120 min) showed the co-elution of sericin and lutein before starting the gradient program of 1 M NaCl buffer. The pigmented-sericin from various treatments was typically resolved into four fractions by the anion exchange column. Decrease in the magnitude of detectable lutein-binding protein fraction was observed, when the treatment time for the pigmented-sericin removal was increased. It was emphasized that the pigmented-sericin probably existed in the degumming solution with polar modification. In generally, lutein is a water-immiscible molecule. However, when lutein existed with sericin, its solubility in aqueous medium was totally modified. This incident probably pointed to the interaction between sericin and lutein existing in the pigmented-sericin solution that resulted in the chemical modification of sericin as well as lutein.

The pigmented-sericin from the degumming process at 121ºC for 15 and 30 min were subjected onto size exclusion. Figure 4.19 shows the elution profiles of the lutein-binding protein on gel filtration chromatography. On Sepharcryl S-200, two peaks with absorption spectra at 460 nm were observed in both treatments. For degumming at 121ºC for 15 min, the first fraction, an apparent molecular mass greater than 158 kDa, was eluted with approximately 9.5% of total lutein (Figure 4.19A). The second fraction showed the molecular mass less than 43 kDa, and accounted for most lutein content. Prolonged heating period for 30 min was affected on the proportion of lutein content in the pigmented-sericin corresponding to previous results of the pigment extraction. As shown in Figure 4.19B, high molecular mass fraction accounted for higher ration of lutein content by 32.0%. These two peaks with

the absorption spectra at 460 nm was associated with the partial characterization of a lutein-binding protein, purified from fifth instar larval midgut of *B. mori* (Jouni and Wells, 1996). Nevertheless, smaller molecular mass of the lutein-binding protein (greater than 125 kDa of the first fraction) was found in the literature values. These were possibly attributed to the difference in the sources of the protein and preparation processes. However, SDS-PAGE profiles of the pigmented-sericin obtained from minimal heat treatment at 85ºC showed the molecular mass of protein compositions corresponding to three main sericins (400, 250 and 150 kDa) in the cocoons of silkworm (*B. mori*) characterized by Takasu, Yamada and Tsuboushi (2002).

CHAPTER V

CONCLUSIONS

The lutein was mainly caroteniods in yellow silk cocoons when compared with other sources leading to lower impurities or higher purity. The predominant (all-*E*)-form of lutein was found in the lutein extract with small presence of its stereoisomer and (*Z*)-geometric isomers. The optimal condition of higher lutein extraction was combination between degumming process and organic solvent extraction. The highest efficiency of lutein extraction was achieved by the use of either tertiary or binary organic solvent systems of hexane, ethanol, ethyl acetate, and acetone. The pigmented-sericin with various proportions of lutein was another part of lutein extract that the lutein-binding protein was characterized. Heat treatment at 121ºC for 15-30 min was appropriate to remove the sericin-lutein complex with a certain proportion of lutein, meanwhile the efficiency of solvent extraction of lutein was enhanced as well.

The lutein might be degraded by light and heat during extraction periods and storage. The degradation rate of lutein associated with the first-order reaction kinetic that fitted well with the two-stage first order kinetic model. Therefore, the extracted lutein should be kept at low temperature without light for extending stability. In addition, the extracted lutein exhibited effective antioxidant activity and chelating agent. This property of lutein may develop for pharmaceutical products and food supplements, and further study to improve the production process.

REFERENCES

Astorg, P. (1997). Food carotenoids and cancer prevention: An overview of current research. **Trends Food Sci. Technol.** 8: 406-413.

Baltschun, D., et al. (1997). Singlet oxygen quenching abilities of carotenoids. **Liebigs Ann.** 9: 1887-1893.

Beatty, S., Nolan, J., Kavanagh, H., and O'Don, O. (2004). Macular pigment optical density and its relationship with serum and dietary levels of lutein and zeaxanthin. **Arch. Biochem. Biophys.** 430: 70-76.

Benzie, I. F. F., and Strain, J. J. (1996). The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": The FRAP assay. **Anal. Biochem.** 239: 70-76.

Bezbradica, D., Milić-Aškrabić, J., Petrović, S. D., and Šiler-Marinković, S. (2005). An investigation of influence of solvent on the degradation kinetics of carotenoids in oil extracts of *Calendula officinalis*. **J. Serb. Chem. Soc.** 70: 115-124.

Bhm, F., Edge, R., Land, E. J., McGarvey, D. J., and Truscott, T. G. (1997). Carotenoids enhance vitamin E antioxidant efficiency. **J. Am. Chem. Soc.** 119: 621-622.

Bhosale, P., and Bernstein, P. S. (2005). Synergistic effects of zeaxanthin and its binding protein in the prevention of lipid membrane oxidation. **Biochim. Biosci. Acta.** 1740: 116-121.

Bhosale, P., and Bernstein, P. S. (2007). Vertebrate and invertebrate carotenoid-binding proteins. **Arch. Biochem. Biophys.** 458: 121-127.

Breithaupt, D. E. (2004). Simultaneous HPLC determination of carotenoids used as food coloring additives: Applicability of accelerated solvent extraction. **Food Chem.** 86: 449-456.

Bone, R. A., Lamdrum, J. T., Dixon, Z., Chen, Y., and Llerena, C. M. (2000). Lutein and zeaxanthin in the eyes, serum and diet of human subjects. **Exp. Eye Res.** 71: 239-245.

Bowen, P. E., Herbst-Espinosa, S. M., Hussain, E. A., and Stacewicz-Sapuntzakis, M. (2002). Esterification does not impair lutein bioavailability in humans. **J. Nutr.** 132: 3668-3673.

Breithaupt, E. D., and Bamedi, A. (2001). Carotenoid esters in vegetables and fruits: A screening with emphasis on *β*-cryptoxanthin esters. **J. Agric. Food Chem.** 49: 2064-2070.

Breithaupt, E. D., Wirt, U., and Bamedi, A. (2002). Differentiation between lutein monoester regioisomers and detection of lutein diesters from marigold flowers (*Tagetes erecta* L.) and several fruits by liquid chromatography-mass spectrometry. **J. Agric. Food Chem.** 50: 66-70.

Britton, G., Liaaen-Jensen, S., and Pfander, H. (eds.). (2004). **Carotenoids Handbook**. Basel: Birkhäuser Verlag.

Burns, J., Fraser, P. D., and Bramley, P. M. (2003). Identification and quantification of carotenoids, tocopherols and chlorophylls in commonly consumed fruits and vegetables. **Phytochem.** 62: 939-947.

Chaiyasit, W., McClements, D. J., and Decker, E. A. (2005). The relationship between the physicochemical properties of antioxidants and their ability to inhibit lipid oxidation in bulk oil and oil-in-water emulsions. **J. Agric. Food Sci.** 53: 4982-4988.

Chandler, L. A., and Schwartz, S. J. (1987). HPLC separation of *cis-trans* carotene isomers in fresh and processed fruits and vegetables. **J. Food Sci.** 3: 669-672.

Chandrasekar, D., Madhusadhana, K., Ramakrishna, S., and Diwan, P. V. (2006). Determination of DPPH free radical scavenging activity by reversed-phase HPLC: A sensitive screening method for polyherbal formulations. **J. Pham. Biomed. Anal.** 40: 460-464.

Chen, B. H., and Chen, Y. Y. (1993). Stability of chlorophylls and carotenoids in sweet potato leaves during microwave heating. **J. Agric. Food Chem.** 41: 1315-1320.

Chen, G., and Djuric, Z. (2001). Carotenoids are degraded by free radicals but do not affect lipid peroxidation in unilamellar liposomes under different oxygen tensions. **FEBS Lett.** 505: 151-154.

Chen, H. E., Peng, H. Y., and Chen, B. H. (1996). Stability of carotenoids and vitamin A during storage of carrot juice. **Food Chem.** 57: 497-503.

Chen, T. M., and Chen, B. H. (1994). Optimization of mobile phases for HPLC of *cis-trans* carotene isomers. **Chromatographia.** 39: 346-354.

Conn, P. F., Schalch, W., and Truscott, T. G. (1991). The singlet oxygen carotenoid interaction. **J. Photochem. Photobiol. B: Biol.** 11: 41-47.

Corts, C., Esteve, M. J., Frgola, A., and Torregrosa, F. (2004). Identification and quantification of carotenoids including geometrical isomers in fruit and vegetable juices by liquid chromatography with ultraviolet-diode array detection. **J. Agric. Food Chem.** 52: 2203-2212.

Craft, N. E., and Soares, J. H. (1992). Relative solubility, stability, and absorptivity of lutein and β-carotene in organic solvents. **J. Agric. Food Chem.** 40: 431-434.

Dachtler, M., Glaser, T., Kohler, K., and Albert, K. (2001). Combined HPLC−MS and HPLC−NMR on-line coupling for the separation and determination of lutein and zeaxanthin stereoisomers in spinach and in retina. **Anal. Chem.** 73: 667-674.

Dachler, M., Kohler, K., and Albert, K. (1998). Reversed-phase high-performance liquid chromatographic identification of lutein and zeaxanthin stereoiosmers in bovine retina using a C_{30} bounded phase. **J. Chromatogr.** 720: 211-216.

Deli, J., Molnár, P., Matus, Z., and Tóth, G. (2001). Carotenoid composition in the fruits of red paprika (*Capsicum annuum* var. *lycopersiciforme rubrum*) during ripening: Biosynthesis of carotenoids in red paprika. **J. Agric. Food Chem.** 49: 1517-1523.

Deli, J., Molnár, M., Osz, A., Tóth, G., and Zsila, F. (2003). Epimerisation of lutein to 30-epilutein in processed foods. **Bioorg. Med. Chem. Lett.** 14: 925-928.

DellaPenna, D. (1999). Carotenoid synthesis and function in plants: Insights from mutant studies in Arabidopsis. **Pure Appl. Chem.** 71: 2205-2212.

Deming, D. M., and Erdman, J. W. (1999). Mammalian carotenoid absorption and metabolism. **Pure Appl. Chem.** 71: 2213-2223.

Desobry, S. A., Netto, F. M., and Labuza, T. P. (1997). Comparison of spray- drying, drum-drying and freeze-drying for *β*-carotene encapsulation and preservation. **J. Food Sci.** 62: 1158-1162.

Dutta, D., Chaudhuri, U. R., and Chakraborty, R. (2005). Structure, health benefits, antioxidant property and processing and storage of carotenoids. **Afr. J. Biotechnol.** 13: 1510-1520.

Emenhiser, C, Sander, L. C., and Schwartz, S. J. (1995). Capability of a polymeric C_{30} stationary phase to resolve *cis-trans* carotenoid isomers in reversed-phase liquid chromatography. **J. Chromatogr. A.** 707: 205-216.

Emenhiser, C., Simunovic, N., Sander, L. C., and Schwartz, S. J. (1996). Separation of geometrical carotenoid isomers in biological extracts using a polymeric C_{30} column in reversed-phase liquid chromatography. **J. Agric. Food Chem.** 44: 3887-3893.

Fabiani, C., Pizzichini, M., Spadoni, M., and Zeddita, G. (1996). Treatment of waste water from silk degumming processes for protein recovery and water reuse. **Desalination.** 105: 1-9.

Feltl, L., Pacakova, V., Stulik, K., and Volka, K. (2005). Reliability of calotenoids analyses: A review. **Current Anal. Chem.** 1: 93-102.

Freddi, G., Mossotti, R., and Innocenti, R. (2003). Degumming of silk fabric with several proteases. **J. Biotechnol.** 106: 101-102.

Furr, H. C., and Cark, R. M. (1997). Intestinal absorption and tissue distribution of carotenoids. **Nutr. Biochem.** 8: 364-377.

Gama, J. J. T., and Sylos, C. M. (2007). Effect of thermal pasteurization and concentration on carotenoid composition of Brazilian Valencia orange juice. **Food Chem.** 100: 1686-1690.

Ganga, G. (2003). **Comprehensive sericulture: Silkworm rearing and silk reeling** (Vol. II). Enfield, NH: Science Publishers.

Granado, F., Olemedilla, B., Gil-Martinez, E., and Blanco, I. (2001). A fast, reliable and low-cost saponification protocol for analysis of carotenoids in vegetables. **J. Food Comp. Anal.** 14: 479-489.

Handelman, G. J., Dratz, E. A., Reay, C. C., and van Kuijk, J. G. (1999). Carotenoids in the human macula and whole retina. **Inves. Ophthalmol. Vis. Sci.** 29: 850-855.

Henry, L. K., Catignani, G. L., and Schwartz, S. J. (1998). Oxidative degradation kinetics of lycopene, lutein, and 9-*cis* and all-*trans* β-carotene. **J. Am. Oil Chem. Soc.** 75: 823-829.

Hirschberg, J., Cohen, M., Harker, M., Lotan, T., Mann, V., and Pecker, I. (1997). Molecular genetics of the carotenoid biosynthesis pathway in plants and algae. **Pure Appi. Chem.** 69: 2151-2158.

Hojnik, M., Škerget, M., and Knez, Ž. (2008). Extraction of lutein from Marigold flower petals-Experimental kinetics and modeling. **LWT - Food Sci. Technol.** 41: 2008-2016.

Hadden, W. L., et al. (1999). Carotenoid composition of marigold (*Tagetes erecta*) flower extract used as nutritional supplement. **J. Agric. Food Chem.** 47: 4189-4194.

Huang, D., Ou, B., and Prior, R. L. (2005). The chemistry behind antioxidant capacity assays. **J. Agric. Food Chem.** 53: 1841-1856.

Humphries, J. M., and Khachik, F. (2003). Distribution of lutein, zeaxanthin, and related geometrical isomers in fruit, vegetables, wheat, and pasta products. **J. Agric. Food Chem.** 51: 1322-1327.

Jang, A., Lee, A., Kim, T. H., Son, J. H., Au, B. J., and Jo, C. (2009). Biological functions of a synthetic compound, octadeca-9,12-dienyl-3,4,5-hydroxy-benzoate, from gallic-linoeic acid ester. **Food Chem.** 112: 369-373.

Jiménez-Escrig, A., Jiménez-Jiménez, I., Sánchez-Moreno, C., and Saura-Calixto, F. (2000). Evaluation of free radical scavenging of dietary carotenoids by the stable radical 2,2-diphenyl-1-picrylhydrazyl. **J. Sci. Food Agric.** 80: 1686-1690.

Jouni, Z. E., and Wells, M. A. (1996). Purification and partial characterization of a lutein-binding protein from the midgut of the silkworm *Bombyx mori*. **J. Biol. Chem.** 271: 14722-14726.

Junghans, A., Sies, H., and Stahl, W. (2001). Macular pigments lutein and zeaxanthin as blue light filter studied in liposomes. **Arch. Biochem. Biophys.** 391: 160-164.

Kaplan, D., Adams, W. W., Farmer, B., and Viney, C. (1994). **Silk polymers: Material science and biotechnology**. Washington, DC: American Chemical Society.

Kato, N., Sato, A., Yamanaka, A., Yamada, H., Fuwa, N., and Nomura, M. (1998). Silk protein, sericin, inhibits lipid peroxidation and tyrosinase activity. **Biosci. Biotechnol. Biochem.** 62: 145-147.

Khachik, F., and Beecher, G. R. (1988). Separation and identification of carotenoids and carotenol fatty acid esters in some squash products by liquid chromatography:

I. Quantification of carotenoids and related esters by HPLC. **J. Agric. Food Chem.** 36: 929-937.

Khachik, F., Bernstein, P. S., and Garland, D. L. (1997). Identification of lutein and zeaxanthin oxidation products in human and monkey retinas. **Invest. Ophthalmol. Vis. Sci.** 38: 1802-1810.

Khachik, F., Englert, G., Beecher, G. R., and Smith, J. C. (1995). Isolation, structural elucidation, and partial synthesis of lutein dehydration products in extracts from human plasma. **J. Chromatogr.** 670: 219-233.

Khachik, F., et al. (1992). Effect of food preparation on qualitative and quantitative distribution of major carotenoid compositions of tomatoes and several green vegetables. **J. Agric. Food Chem.** 40: 390-398.

Khachik, F., Moura, F. F., Zhao, D., Aebischer, C., and Bernstein, P. S. Transformations of selected carotenoids in plasma, liver, and ocular tissues of humans and in nonprimate animal models. **Invest. Ophthalmol. Vis. Sci.** 43: 3383-3392.

Kim, H., Chen, F., Wang, X., Chung, H. Y., and Jin, Z. (2005). Evaluation of antioxidant activity of Vetiver (*Vetiveria zizanioides* L.) oil and identification of its antioxidant constituents. **J. Agric. Food Chem.** 53: 7691-7695.

Kimura, M., and Rodriguez-Amaya, D. B. A. (2002). Scheme for obtaining standards and HPLC quantification of leafy vegetable carotenoids. **Food Chem.** 78: 389-398.

Kimura, M., Kobori, C. N., Rodriguez-Amaya, D. B. A., and Nestel, P. (2007). Screening and HPLC methods for carotenoids in sweetpotato, cassava and maize for plant breeding trails. **Food Chem.** 100: 1734-1746.

Konings, E. J. M., and Roomans, H. H. S. (1997). Evaluation and validation of an LC method for the analysis of carotenoids in vegetables and fruit. **Food Chem.** 59: 599-603.

Krinsky, N. I., and Johnson, E. J. (2005). Carotenoid actions and their relation to health and disease. **Mol. Aspects Med.** 26: 459-516.

Krinsky, N. I. (1998). The antioxidant and biological properties of the carotenoids. **Ann. N. Y. Acad. Sci.** 854: 443-447.

Krinsky, N. I., and Yeum, K. (2003). Carotenoid-radical interaction. **Biochem. Biophys. Res. Commun.** 305: 754-760.

Lakshminarayana, R., Aruna, G., Sangeetha, R. K., Bhaskar, N., Divakar, S., and Baskaran, V. (2008). Possible degradation/biotransformation of lutein *in vitro* and *in vivo*: Isolation and structural elucidation of lutein metabolites by HPLC and LC-MS (atmospheric pressure chemical ionization). **Free radic. Biol. Med.** 45: 982-993.

Landrum, J. T., and Bone, R. A. (2001). Lutein, zeaxanthin, and the macular pigment. **Arch. Biochem. Biophys.** 385: 28-40.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. **Nature.** 227: 680-685.

Lamoolphak, W., De Eknamkul, W., and Shotipruk, A. (2008). Hydrothermal production and characterization of protein and amino acids from silk waste. **Bioresour. Technol.** 99: 7678-7685.

Lavecchia, R., and Zuorro, A. (2006). Shelf stability of lutein from marigold (*Tagetes erecta* L.) flowers in vegetable oils. **Chem. Technol.** 80: 1-6.

Lee, H. S., and Coates, G. A. (2003). Effect of thermal pasteurization on Valencia orange juice color and pigments. **Lebensm.-Wiss. Technol.** 36: 153-156.

Lefsrud, M., Kopsell, D., Wenzel, A., and Sheehan, J. (2007). Changes in kale (*Brassica oleracea* L. var. *acephala*) carotenoid and chlorophyll pigment concentrations during leaf ontogeny. **Sci. Hort.** 112: 136-141.

Li, W., Gao, Y., Zhao, J., and Wang, Q. (2007). Phenolic, flavonoid, and lutein ester content and antioxidant activity of 11 cultivars of Chinese marigold. **J. Agric. Food Chem.** 55: 8478-8484.

Li, X., and Han, L. (2008). Iron(II)-induced isomerization of (all-*E*)-xanthophyll pigments lutein, zeaxanthin, and *β*-cryptoxanthin in acetone. **Eur. Food Res. Technol.** 227: 1307-1313.

Lin, C. H., and Chen, B. H. (2003). Determination of carotenoids in tomato juice by liquid chromatography. **J. Chromatogr. A.** 1012: 103-109.

Lin, C. H., and Chen, B. H. (2005). Stability of carotenoids in tomato juice during storage. **Food Chem.** 90: 837-846.

Liu, Y., Perera, C. O., and Suresh, V. (2007). Comparison of three chosen vegetables with others from South East Asia for their lutein and zeaxanthin content. **Food Chem.** 101: 1533-1539.

Marx, M., Schieber, A., and Carle, R. (2003). Effects of thermal processing on *trans-cis*-isomerization of *β*-carotene in carrot juices and carotene-containing preparations. **Food Chem.** 83: 609-617.

Martin, H. D., et al. (1999). Chemistry of carotenoid oxidation and free radical reaction. **Pure Appl. Chem.** 71: 2253-2262.

Matus, Z., Deli, J., and Szabolcs, J. (1991). Carotenoid composition of yellow pepper during ripening: Isolation of *β*-cryptoxanthin 5,6-epoxide. **J. Agric. Food Chem.** 39: 1907-1914.

McCall, J. M. (1975). Liquid-liquid partition coefficients by high-pressure liquid chromatography. **J. Med. Chem.** 18: 549-552.

McInerney, J. K., Seccafien, C. A., Stewart, C. M., and Bird, A. R. (2007). Effects of high pressure processing on antioxidant activity, and total carotenoid content and availability, in vegetables. **Innov. Food Sci. Emerg. Technol.** 8: 543-548.

McNulty, H. P., Byun, J., Lockwood, S. F., Jacob, R. F., and Mason, R. P. (2007). Differential effects of carotenoids on lipid peroxidation due to membrane interactions: X-ray diffraction analysis. **Biochim. Biophys. Acta.** 1768: 167-174.

Mercadante, A. Z., and Rodriguez-Amaya, D. B. A. (1991). Carotenoid composition of a leafy vegetable in relation to some agricultural variables. **J. Agric. Food Chem.** 39: 1094-1097.

Meléndez-Martínez, A. J., Britton, G., Vicario, I. M., and Heredia, F. J. (2006). HPLC analysis of geometrical isomers of lutein epoxide isolated from dandelion (*Taraxacum officinale* F. Weber ex Wiggers). **Phytochem.** 67: 771-777.

Meléndez-Martínez, A. J., Britton, G., Vicario, I. M., and Heredia, E. J. (2005). Identification of isolutein (lutein epoxide) as *cis*-antheraxanthin in orange juice. **J. Agric. Food Chem.** 53: 9369-9373.

Milardović, S., Ivekovic, D., and Grabaric, B. S. (2006). A novel amperometric method for antioxidant activity determination using DPPH· free radical. **Bioelectrochem.** 68: 175-180.

Milanowska, J., and Gruszecki, W. I. (2005). Heat-induced and light-induced isomerization of the xanthophyll pigment zeaxanthin. **J. Photochem. Photobiol. B: Biol.** 80: 178-186.

Miller, N. J., Sampson, J., Candeias, L. P., Bramley, P. M., and Rice-Evants, C. A. (1996). Antioxidant activities of carotenes and xanthophylls. **FEBS Lett.** 384: 240-242.

Minguez-Mosquera, M. S., and Gandul-Rojas, B. (1994). Mechanism and kinetics of carotenoid degradation during the processing of green table olives. **J. Agric. Food. Chem.** 42: 1551-1554.

Molnár, P. (2009). Research of the (*E*/*Z*)-isomerization of carotenoids in Pécs since the 1970s. **Arch. Biochem. Biophys.** 483: 156-164.

Mortensen, A., and Skibsted, L. H. (1997). Important of carotenoid structure in radical-scavenging reactions. **J. Agric. Food. Chem.** 45: 2970-2977.

Navarrete-Bolaňos, J. L., Rangel-Cruz, C. L., Jiménez-Islas, H., Botello-Alvarez, E., and Rico-Martínez, R. (2005). Pre-treatment effects on the extraction efficiency of xanthophylls from marigold flower (*Tagetes erecta*) using hexane. **Food Res. Inter.** 38: 159-165.

Nelis, H. J. C. F., and De Leenheer, A. P. (1983). Isocratic nonaqueous reversed-phase liquid chromatography of carotenoids. **Anal. Chem.** 55: 270-275.

Olmedilla, B., Granado, F., Blanco, I., and Vaquero, M. (2003). Lutein, but not α-tocopherol, supplementation improves visual function in patients with age-related cataracts: A 2-y double-blind, placebo-controlled pilot study. **Nutr.** 19: 21-24.

Piccaglia, R., Marotti, M., and Grandi, S. (1998). Lutein and lutein ester content in different types of *Tagetes patula* and *T. erecta*. **Ind. Crop. Pro.** 8: 45-51.

Pérez-Gálvez, A., and Mínguez-Mosquera, M. I. (2002). Degradation of non-esterified and esterified xanthophylls by free radicals. **Biochim. Biophys. Acta.** 1569: 31-34.

Pérez-Gálvez, A., and Mínguez-Mosquera, M. I. (2005). Esterification of xanthophylls and its effect on chemical behavior and bioavailability of carotenoids in the human. **Nutr. Res.** 25: 631- 640.

Polykov, N. E., Leshina, T. V., Konovalova, T. A., and Kispert, L. D. (2001). Carotenoids as scavenging of free radicals in a Fenton reaction: Antioxidants or Pro-oxidants?. **Free Radic. Biol. Med.** 31: 398-404.

Pott, I., Marx, M., Neidhart, S., Mühlbauer, W., and Carle, R. (2003). Quantitative determination of *β*-carotene stereoisomers in fresh, dried, and solar-dried mangoes (*Mangifera indica* L.). **J. Agric. Food Chem.** 51: 4527-4531.

Prommuak, C., De Eknamkul, W., and Shotipruk, A. (2008). Extraction of flavonoids from Thai silk waste and antioxidant activity of extracts. **Sep. Purif. Technol.** 62: 444-448.

Rajendran, V., Pu, Y. S., and Chen, B. H. (2005). An improved HPLC method for determination of carotenoids in human serum. **J. Chromatogr. B.** 824: 99-106.

Razungles, A. J., Babic, I., Sapis, J. C., and Bayonove, C. L. (1996). Particular behavior of epoxy xanthophylls during veraison and maturation of grape. **J. Agric. Food Chem.** 44: 3821-3825.

Rodríguez-Amaya, D. B. A. (2001). **A guide to carotenoid analysis in foods**. Washington, DC: ILSI Press.

Rodriguez-Amaya, D. B. A., and Kimura, M. (2004). **HarvestPlus handbook for carotenoid analysis**. Washington, DC: IFPRI.

Rouseff, R., and Hofsommer, L. R. (1996). Application of diode array detector with a C_{30} reversed phase column for the separation and identification of saponified orange juice carotenoids. **J. Agric. Food Chem.** 44: 2174-2181.

Semba, R. D., and Dagnelie, G. (2003). Are lutein and zeaxanthin conditionally essential nutrients for eye health?. **Med. Hypotheses.** 61: 465-472.

Sánchez-Moreno, C., Larrauri, J., and Saura-Calixto, F. A procedure to measure the antiradical efficiency of polyphenols. **J. Sci. Food Agric.** 76: 270-276.

Sánchez-Moreno, C., Plaza, L., De Ancos, B., and Cano, M. P. (2006). Nutritional characterization of commercial traditional pasturised tomato juices: Carotenoids, vitamin C and radical-scavenging capacity. **Food Chem.** 98: 749-759.

Santas, J., Carbó, R., Gordon, M. H., and Almajano, M. P. (2008). Comparison of the antioxidant activity of two Spanish onion varieties. **Food Chem.** 107: 1210-1216.

Sasaki, M., Yamada, H., and Kato, N. (2000). Consumption of silk protein, sericin elevates intestinal absorption of zinc, iron, magnesium, and calcium in rats. **Nutr. Res.** 20: 1505-1511.

Schäffer, M. W., Roy, S. S., Mukherjee, S., and Das, S. K. (2008). Identification of lutein, a dietary antioxidant carotenoid in guinea pig tissues. **Biochem. Biophys. Res. Commun.** 374: 378-381.

Schieber, A., and Carle, R. (2005). Occurrence of carotenoid *cis*-isomers in food: Technological, analytical, and nutritional implications. **Trends Food Sci. Technol.** 16: 416-422.

Schlatterer, J., and Breithaupt, D. E. (2006). Xanthophylls in commercial egg yolks: Quantification and identification by HPLC and LC-(APCI)MS using a C_{30} phase. **J. Agric. Food Chem.** 54: 2267-2273.

Seves, A., Romanò, M., Maifreni, T., Sora, S., and Ciferri, O. (1998). The microbial degradation of silk: A laboratory investigation. **Int. Biodeterior. Biodegrad.** 42: 203-211.

Shafaa, M. W. I., Diehl, H. A., and Socaciu, C. (2007). The solubilisation pattern of lutein, zeaxanthin, canthaxanthin and *β*-carotene differ characteristically in liposomes, liver microsomes and retinal epithelial cells. **Biophys. Chem.** 129: 111-119.

Shao, A. (2001). The role of lutein in human health: A review. **JANA.** 4: 7-19.

Smith, P. K., et al. (1985). Measurement of protein using bicinchoninic acid. **Anal. Biochem.** 150: 76-85.

Socaciu, C. (ed.). (2007). **Food Colorants Chemical and Functional Properties**. Boca Ration, Fla.: CRC Press.

Sommerburg, O., Keunen, J., Bird, A., and Kuijk, F. (1998). Fruits and vegetables that are sources for lutein and zeaxanthin: The macular pigment in human eyes. **Br. J. Ophthalmol.** 82: 907-910.

Stahl, W., Junghans, A., De Boer, B., Driomina, E. S., Briviba, K., and Sies, H. (1998). Carotenoid mixtures protect multilamellar liposomes against oxidative damage: Synergistic effects of lycopene and lutein. **FEBS Lett.** 427: 305-308.

Stahl, W., and Sies, H. (2003). Antioxidant activity of carotenoids**. Mol. Asp. Med.** 24: 345-351.

Stahl, W., and Sies, H. (2005). Bioactivity and protective effects of natural carotenoids. **Biochim. et Biophys. Acta.** 1740: 101-107.

Subagio, A, and Morita, N. (2001). No effect of esterification with faty acid on antioxidant activity of lutein. **Food Res. Int.** 34: 315-320.

Subagio, A., Wakaki, H., and Morita, N. (1999). Stability of lutein and its myristate ester. **Biosci. Biotechnol. Biochem.** 63: 1784-1786.

Sujak, A., Gabrielska, J., Grudziński, W., Borc, R., Mazurek, P., and Gruszeki, W. I. (1999). Lutein and zeaxanthin as protectors of lipid membranes against oxidative damage: The structural aspects. **Arch. Biochem. Biophys.** 371: 301-307.

Sujak, A., Okulski, W., and Gruszecki, W. I. (2000). Organization of xanthophylls pigments lutein and zeaxanthin in lipid membranes formed with dipalmitoyl-phosphatidylcholine. **Biochim. Biophys. Acta.** 1509: 255-263.

Szabo, M. R., Iditoiu, C., Chambre, D., and Lupea, A. X. (2007). Improved DPPH· determination for antioxidant activity spectrophotometric assay. **Chem. Pap.** 61: 214-216.

Tabunoki, H., et al. (2004). A carotenoid-binding protein (CBP) plays a crucial role in cocoon pigmentation of silkworm (*Bombyx mori*) larvae. **FEBS Lett.** 567: 175-178.

Tabunoki, H., et al. (2002). Isolation, characterization, and cDNA sequence of a carotenoid binding protein from the silk gland of *Bombyx mori* larvae. **J. Biol. Chem.** 277: 32133-32140.

Tai, C. Y., and Chen, B. H. (2000). Analysis and stability of carotenoids in the flowers of daylily (*Hemerocallis disticha*) as affected by various treatments. **J. Agric. Food Chem.** 48: 5962-5968.

Takasu, Y., Yamada, H., and Tsuboushi, K. (2002). Isolation of three main sericin components from the cocoons of the silkworm, *Bombyx mori*. **Biosci. Biotechnol. Biochem.** 12: 2715-2718.

Tang, Y. C., and Chen, B. H. (2000). Pigment change of freeze-dried carotenoid powder during storage. **Food Chem.** 69: 11-17.

Teramoto, H., and Miyazawa, A. T. (2006). Native structure and degradation pattern of silk sericin studied by 13C NMR spectroscopy. **Macromol.** 39: 6-8.

Thaipong, K., Boonprakob, U., Crosby, K., Cisneros-Zevallos, L., and Byrne, D. H. (2006). Comparison of ABTS, DPPH, FRAP, and ORAC assays for estimating antioxidant activity from guava fruit extracts. **J. Food Comp. Anal.** 19: 669-675.

Tsao, R., Yang, R., Young, J. C., Zhu, H., and Manolis, T. (2004). Separation of geometric isomers of native lutein diesters in marigold (*Tagetes erecta* L.) by high-performance liquid chromatography-mass spectrometry. **J. Chromatogr. A.** 1045: 65-70.

Tsao, R., and Yang, R. (2006). Lutein in selected Canadian crops and agri-food processing by-products and purification by high-speed counter-current chromatography. **J. Chromatogr. A.** 1112: 202-208.

Tsuchida, K., Arai, M., Tanaka, Y., Ishihara, R., Ryan, O. R., and Maekawa, H. (1998). Lipid transfer particle catalyzes transfer of carotenoids between lipophorins of *Bombyx mori*. **Insect Biochem. Mol. Biol.** 28: 927-934.

Tsuchida, K., et al. (2004a). Characterization of the carotenoid-binding protein of the Y-gene dominant mutants of *Bombyx mori*. **J. Insect Physiol.** 50: 363-372.

Tsuchida, K., et al. (2004b). The basis for colorless hemolymph and cocoons in the Y-gene recessive *Bombyx mori* mutants: A defect in the cellular uptake of carotenoids. **J. Insect Physiol.** 50: 975-983.

Updike, A. A., and Schwartz, S. J. (2003). Thermal processing of vegetables increases *cis* isomers of lutein and zeaxanthin. **J. Agric. Food Chem.** 51: 6184-6190.

Vaithanomsat, P., and Kitpreechavanich, V. (2008). Sericin separation from silk degumming wastewater. **Sep. Purif. Technol.** 59: 129-133.

Veneria, E., et al. (2008). Assessment of the nutritional values of genetically modified wheat, corn, and tomato crops. **J. Agric. Food. Chem.** 56: 9206-9214.

Vishnevetsky, M., Ovadis, M., and Vainstein, A. (1999). Carotenoid sequestration in plants: The role of carotenoid associated proteins. **Trends Plant Sci.** 4: 232-235.

Wang, M., et al. (2006). Antioxidant activity, mutagenicity/anti-mutageneticity, and clastogenicity/anti-clastogenicity of lutein from marigold flowers. **Food Chem. Toxicol.** 44: 1522-1529.

Winkler, B. S., Boulton, M. E., Gottsch, J. D., and Sternberg, P. (1999). Oxidative damage and age-related macular degeneration. **Mol. Vis.** 3: 5-32.

Woodall, A. A., Lee, S. W., Weesie, R. J., Jackson, M. J., and Britton, G. (1997). Oxidation of carotenoids by free radicals: Relationship between structure and reactivity. **Biochim. Biophys. Acta.** 1336: 33-42.

Wrolstad, R. E., et al. (2005). **Handbook of food analytical chemistry: Pigments, colorants, flavors, texture, and bioactive food components**. New Jersey: Wiley-Interscience.

Wu, J., Wang, Z., and Xu, S. (2007). Preparation and characterization of sericin powder extracted from silk industry wastewater. **Food Chem.** 103: 1255-1262.

Wu, J., Wang, Z., and Xu, S. (2008). Enzymatic production of bioactive peptides from sericin recovered from silk industry wastewater. **Process Biochem.** 43: 480-487.

Xin-yu, J., Long-sheng, C., and Chun-shan, Z. (2005). Lutein and lutein esters in marigold flowers by high performance chromatography. **J. Cent. South Univ. Technol.** 12: 306-308.

Yemelyanov, A. Y., Katz, N. B., and Bernstein, P. S. (2001). Ligand-binding characterization of xanthophyll carotenoids to solubilized membrane proteins derived from human retina. **Exp. Eye Res.** 72: 381-392.

Yen, W. J., and Chen, B. H. (1995). Isolation of xanthophylls from Taiwanese orange peek and their effects on the oxidation stability of soybean oil. **Food Chem.** 53: 417-425.

REFERENCES

Ahmed, S. S., Lott, M. N., and Marcus, D. M. (2005). The macular xanthophylls. **Surv. Ophthalmol.** 50: 183-193.

Ak, T., and Gulcin, I. (2008). Antioxidant and radical scavenging properties of curcumin. **Chem. Biol. Interact.** 174: 27-37.

Alves-Rodrigures, A., and Shao, A. (2004). The science behind lutein. **Toxicol. Lett.** 150: 57-83.

Aman, R., Bayha, S., Carle, R., and Schieber, A. (2004). Determination of carotenoid stereoisomers in commercial dietary supplements by high-performance liquid chromatography. **J. Agri. Food Chem.** 52: 6086-6090.

Aman, R., Biehl, J., Carle, R., Conrad, J., Beifuss, U., and Schieber, A. (2005). Application of HPLC coupled with DAD, APcI-MS and NMR to the analysis of lutein and zeaxanthin stereoisomers in thermally processed vegetables. **Anal. Nutri. Clin. Methods.** 92: 753-763.

Aman, R., Schieber, A., and Carle, R. (2005). Effects of heating and illumination on *trans-cis* isomerization and degradation of *β*-carotene and lutein in isolated spinach chloroplasts. **J. Agric. Food Chem.** 53: 9512-9518.

Ancos, B., Gonzalez, E., and Cano, M. P. (2000). Effect of high pressure treatment on the carotenoid composition and the radical scavenging ability of Persimmon fruit purees. **J. Agric. Food Chem.** 48: 3542-3548.

Antolovich, M., Prenzler, P. D., Patsalids, E., McDonald, S., and Robards, K. (2002). Methods for testing antioxidant activity. **Analyst.** 127: 183-189.

You, L., Zhao, M., Cui, C., Zhao, H., and Yang, B. (2009). Effect of degree of hydrolysis on the antioxidant activity of loach (*Misgurnus anguillicaudatus*) protein hydrolysates. **IFSET.** 10: 235-240.

Young, J. C., Abdel-Aal, E. M., Rabalski, I., and Blackwell, B. (2007). Identification of synthetic regioisomeric lutein esters and their quantification in a commercial lutein supplement. **J. Agric. Food. Chem.** 55: 4965-4972.

Zang, L., Sommerberg, O., and Frederik, J. G. M. (1997). Absorbance changes of carotenoids in different solvents. **Free Radic. Biol. Med.** 23: 1086-1089.

Zaripheh, S., and Erdman, J. W. (2002). Factors that influence the bioavailability of xanthophylls. **J. Nutr.** 132: 531S-534S.

Zhang, H., Chen, F., Wang, X., and Yao, H. (2006). Evaluation of antioxidant activity of parsley (*Petroselinum crispum*) essential oil and identification of its antioxidant constituents. **Food Res. Int.** 39: 833-839.

Zhaorigetu, S., Sasaki, M., Watanabe, H., and Kato, N. (2001). Supplemental silk protein, sericin, suppresses colon tumorigenesis in 1,2-dimethylhydrazine-treated mice by reducing oxidative stress and cell proliferation. **Biosci. Biotechnol. Biochem.** 65: 2181-2186.

Zhaorigetu, S., Yanaka, N., Sasaki, M., Watanabe, H., and Kato, N. (2003). Inhibitory effects of silk protein, sericin on UVB-induced acute damage and tumor promotion by reducing oxidative stress in the skin of hairless mouse. **J. Photochem. Photobiol. B.** 71: 11-17.

BIOGRAPHY

Potchanee Kaewkumsan was born on October 12, 1977 in Sakon Nakhon Province, Thailand. She studied for her high school diploma at Bunlua Wittayanusorn School (1991-1996) in Nakhon Ratchasima Province, Thailand. In 2000, she received her Bachelor of Science in Food Technology from Suranaree University of Technology, Nakhon Ratchasima. After graduation (2000-2004), she took a quality control supervisor position at Thai Union Frozen Products PCL, Samutsakhon Province, Thailand. She pursued a Master of Science degree in Food Technology under Asst. Prof. Dr. Manote Sutheerawattananonda at Suranaree University of Technology in 2004. During graduate study, she worked as a research assistant who was responsible for two research projects; lutein from yellow silk *Bombyx mori* cocoons, granted by the Commission on Higher Education (2006-2007) and the Agricultural Research Development Agency (Public Organization) (2008-2009). Additionally, she obtained an opportunity to participate in research works regarding to silk protein sericin, supported by the Thailand Research Fund (2006-2007) and the National Research Council of Thailand (2008). From the effort she put into her course research and those research responsibilities, she has co-submitted the invention of processes for lutein and sericin-lutein complex production from yellow silk *B. mori* cocoons to patent as a co-inventor with the Suranaree University of Technology in 2008.