การสกัดสารฟลาโวนอยด์และแคโรทีนอยด์จากเศษไหมไทย
และฤทธิ์ในการต่อต้านอนุมูลอิสระของสารสกัด

นางสาวฉัตรทิพย์ พรหมหมวก

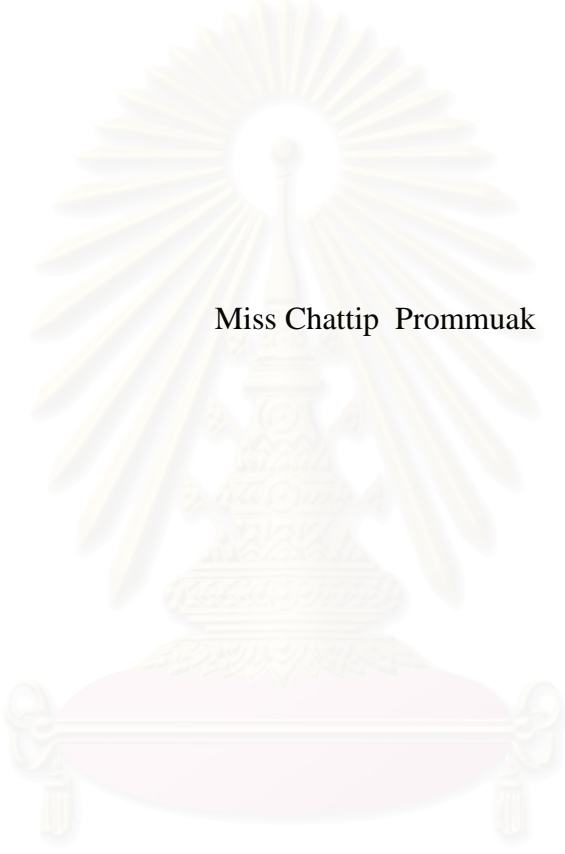วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิศวกรรมศาสตรมหาบัณฑิต
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EXTRACTION OF SILK FLAVONOIDS AND CAROTENOIDS FROM THAI
SILK WASTE AND THEIR ANTIOXIDANT ACTIVITIES

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A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Engineering Program in Chemical Engineering
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Thesis Title	EXTRACTION OF SILK FLAVONOIDS AND CAROTENOIDS FROM THAI SILK WASTE AND THEIR ANTIOXIDANT ACTIVITIES
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ฉัตรทิพย์ พรหมหมวก : การสกัดสารฟลาโวนอยด์และแคโรทีนอยด์จากเศษไหมไทยและฤทธิ์ในการต่อต้านอนุมูลอิสระของสารสกัด (EXTRACTION OF SILK FLAVONOIDS AND CAROTENOIDS FROM THAI SILK WASTE AND THEIR ANTIOXIDANT ACTIVITIES) อาจารย์ที่ปรึกษา : ผศ.ดร. อาทิวรรณ โชติพฤกษ์,78 หน้า.

ในปัจจุบัน มีการศึกษาวิธีการสกัด ตลอดจนการนำเอาโปรตีนจากไหมไปประยุกต์ใช้กันอย่างกว้างขวาง งานวิจัยนี้มุ่งที่จะศึกษาอีกส่วนหนึ่งของไหมซึ่งมีความน่าสนใจ ไม่แพ้ในส่วนของโปรตีนไหม นั่นคือ ส่วนที่ทำให้ไหมมีสีสันต่างๆ หรือที่เรียกว่า พิกเมนต์ พิกเมนต์ในไหมนี้ประกอบด้วยสารจำพวกแคโรทีนอยด์และสารในกลุ่มฟีโนลิกที่เรียกว่าฟลาโวนอยด์ซึ่งมีฤทธิ์ในการต่อต้านอนุมูลอิสระ จากการทดลองหาปริมาณแคโรทีนอยด์และ ฟลาโวนอยด์โดยรวมที่มีอยู่ทั้งหมดในเศษไหมของไหมไทยสีเหลือง พันธุ์นางน้อย โดยการสกัดซ้ำ 5 ครั้ง ด้วยเอทานอล พบว่าสารสกัดดังกล่าวมีปริมาณแคโรทีนอยด์และฟลาโวนอยด์โดยรวม 0.7 และ 5.1 มิลลิกรัมต่อกรัมของไหมแห้ง ตามลำดับ ซึ่งประมาณ 60% ของแคโรทีนอยด์ และ กว่า 70% ของฟลาโวนอยด์ จะอยู่ที่ชั้นของไฟโบรอิน (โปรตีนส่วนแกนกลางของไหม) และจากการวิเคราะห์ด้วยเครื่องโครมาโทกราฟีของเหลวสมรรถนะสูง พบว่าส่วนประกอบหลักของสารสกัดนี้คือสารที่มีความสามารถในการปกป้องดวงตาที่เรียกว่า ลูทีน

เนื่องจากประโยชน์ของสารสกัดนี้คือมีความสามารถในด้านยา จึงได้ทำการศึกษาตัวทำละลายสองชนิด คือ เอทานอลและน้ำที่ภาวะกึ่งวิกฤต เพื่อเลือกตัวทำละลายที่มีความปลอดภัย และเหมาะสมต่อการสกัดพิกเมนต์เหล่านี้ จากผลการทดลอง พบว่า เอทานอลเป็นตัวทำละลายที่เหมาะสมต่อการสกัดแคโรทีนอยด์ ในขณะที่น้ำกึ่งวิกฤตเป็นตัวทำละลายที่เหมาะสมต่อการสกัดฟลาโวนอยด์ จึงได้ทำการทดลองสกัดเศษไหมด้วยเอทานอลที่อุณหภูมิ 50-79 องศาเซลเซียส และใช้เวลาในการสกัดตั้งแต่ 2-12 ชั่วโมง เพื่อศึกษาศึกษาอิทธิพลของอุณหภูมิและเวลาในการสกัดต่อปริมาณสารแคโรทีนอยด์ที่สกัดได้ พบว่า ปริมาณสารแคโรทีนอยด์จะเพิ่มขึ้นเมื่ออุณหภูมิและระยะเวลาที่ใช้ในการสกัดเพิ่มขึ้น นอกจากนี้ ได้ทำการทดลองสกัดไหมด้วยน้ำกึ่งวิกฤตที่อุณหภูมิ 120-150 องศาเซลเซียส และระยะเวลาที่ใช้ในการสกัด 10-60 นาที เพื่อศึกษาอิทธิพลของอุณหภูมิน้ำกึ่งวิกฤตตลอดจนระยะเวลาที่ใช้ในการสกัดต่อปริมาณสารฟลาโวนอยด์ที่สกัดได้ พบว่า ปริมาณสารฟลาโวนอยด์จะลดลงเมื่ออุณหภูมิของน้ำกึ่งวิกฤตและระยะเวลาที่ใช้ในการสกัดเพิ่มขึ้น

สำหรับการศึกษาความสามารถในการต่อต้านอนุมูลอิสระของสารสกัดจากไหม พบว่า ความสามารถในการต่อต้านอนุมูลอิสระนั้นเป็นผลมาจากพิกเมนต์ ซึ่งสามารถยับยั้งการทำงานของอนุมูลอิสระลงได้ 50% ด้วยความเข้มข้นของสารสกัดเพียง 15.5-23.3 ไมโครกรัมต่อมิลลิตร

ภาควิชา.........วิศวกรรมเคมี...............	ลายมือชื่อนิสิต...ฉัตรทิพย์ พรหมหมวก...	
สาขาวิชา.........วิศวกรรมเคมี...............	ลายมือชื่ออาจารย์ที่ปรึกษา...............	
ปีการศึกษา.......2550..................	ลายมือชื่ออาจารย์ที่ปรึกษาร่วม...............	

4870652521   : MAJOR CHEMICAL ENGINEERING DEPARTMENT

KEY WORD: SILK / CAROTENOID / FLAVONOID / SUBCRITICAL WATER / ETHANOLIC EXTRACTION / ANTIOXIDANT ACTIVITY

CHATTIP PROMMUAK : EXTRACTION OF SILK FLAVONOIDS AND CAROTENOIDS FROM THAI SILK WASTE AND THEIR ANTIOXIDANT ACTIVITIES. THESIS ADVISOR : ASST. PROF. ARTIWAN SHOTIPRUK, PhD., 78 pp.

At present, there have been widespread studies on the extraction of silk proteins as well as their applications. This thesis aims to study silk pigments, the other component of the silk, which was interesting no less than its protein. Silk pigments are composed of antioxidative substances such as carotenoids and phenolic compounds, particularly flavonoids. The results in this study showed that the complete removal of the pigments of Thai yellow Nangnoi silk waste, could be achieved by repeated batch extraction 5 time with ethanol at 80°C in a closed pressure vessel. The extracts exhibited the amounts of total carotenoids and flavonoids of 0.7 and 5.1 mg/g dry weight, respectively. Of these amounts, more than 60% of carotenoids contents and 70% of flavonoids contents were located on silk fibroin layer (an inner core protein). The constituents of this extract were then analyzed using high performance liquid chromatography (HPLC) and it was found that the carotenoid called lutein is the major component of the extract.

Due to the fact that carotenoids and flavonoids are associated with the medicinal functions, we investigated extraction of these pigments using two benign solvents, which are ethanol and subcritical water to determine the suitability of the solvents for extraction of these pigments. The experimental results revealed that ethanol is the suitable solvent for extraction of carotenoids while subcritical water is suitable for flavonoids extraction. For extraction of caroteinoids, ethanolic was used as extraction solvent to determine the effect of temperature (50-79°C) and extraction time (2-12 hours), and the result exhibited that the amount of carotenoids increased with increasing temperature and extraction time. For flavonoids, subcritical water extraction was carried out at 120-150°C for 10-60 min to determine the effects of subcritical water temperatures and extraction times on the yield, and the results showed that the amount of flavonoids decreased with the increasing subcritical water temperature and the extraction time due to decomposition at such conditions.

Besides the amount of the pigments, the antioxidant activity of the silk extracts were determined and it was found that the concentration of the silk pigment extract that exhibit the 50% reduction of 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid), (ABTS) free radicals was as small as 15.6-23.3 µg/ml

Department.......Chemical Engineering.........Student's signature........

Field of study.....Chemical Engineering.........Advisor's signature........

Academic year......2007.........................Co-advisor's signature........

ACKNOWLEDGMENTS

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CHAPTER I

INTRODUCTION

1.1 Rationale

Silk waste is the part of the cocoons of the silkworms *(Bombyx mori)* that is difficult to unreel. In Thailand, a large amount (36.6 tons) of this by-product is produced annually. Despite their unsuitability for silk textile production, the composition of silk waste is similar to that of good silk. It is composed of 75-83% of fibroin (an inner core protein), which is surrounded by a glue-like protein called sericin (17-25%). Other components include waxes, hydrocarbons, and pigments. Sericin has been found to have antioxidant as well as medical activities, and is used for various application such as cosmetics, functional biomaterials, and medicines. The more fibrous fibroin protein is utilized as functional biomaterial especially for tissue engineering. Several studies are currently being conducted on extraction, fabrication, and utilization of silk proteins [Mizoguchi et al., 1991, Nomura et al., 1995, Kato et al., 1998, Yoshii et al., 2000, Sarovart et al., 2003 and Meinel et al., 2004]. In many applications, sericin and fibroin need to be made into soluble forms. For sericin, solubilization could be readily achieved in boiling water, while solubilization of fibroin, on the other hand, is an acid or alkali catalyzed process. Moreover, fibroin could also be solubilized in mixtures of aqueous solution of salts and organic solvents such as $CaCl_2/H_2O/EtOH$ [Yeo et al., 2003]. However, toxic chemicals and severe conditions used make the process unfavorable. The development of an effective process based on enzymes as active agents would entail savings in terms of water, energy, chemicals, effluent treatment [Gulrajani, 1996] However, the higher cost of enzymes themselves has so far limited the development of industrial processes. This led us to examine a non-catalytic hydrothermal process for solubilization of sericin and fibroin from silk waste into useful protein and amino acids [Lamoolphak et al., 2006]. The results demonstrated that subcritical water could potentially be used for extraction of silk sericin and fibroin proteins.

Other than sericin and fibroin, silk, particularly the native silk like Nangnoi silk cultivated widely in Thailand, contains a significant amount of pigments. The

pigments present in silk are mostly associated with carotenoids and flavonoids [Tabunoki et al., 2004 and Tamura et al., 2001]. Carotenoids are the most widespread pigments in nature and are the compounds responsible for the remarkable golden yellow color of Thai silk. The major carotenoid in the yellow colored silk was reported to be lutein (80% of total carotenoids). Other silk pigments include phenolic compounds particularly flavonoids which come from mulberry leaves, the only food of *Bombyx mori* [Oku, 1934; Hayashiya et al., 1959]. Both carotenoids and flavonoids are pigments generally found in fruits and vegetables, and are remarkably reported as a highly efficient antioxidant compounds. Flavonoids have several pharmacological activities such as cardiotonic, anti-inflammatory, and anti-microbial activities [Itoigawa et al., 1999, Shahidi et al., 1998 and Wild et al., 1969], while carotenoids are associated with the prevention of the diseases such as cancer and cardiovascular disease [Kohlmier et al., 1995]. The oxygenated silk carotenoid (xanthophyll), lutein, has been reported as an excellent agent to protect against vision loss [Hammond et al., 1997 and Wooten et al., 2002].

Thongphasuk et al. (2005) recently showed that the antioxidant activity of silk extract were not only resulted by sericin but also by silk pigments. That the authors reported that the value of IC_{50} of ethanolic extract, composed mostly of silk pigments, was lower than that of water extracts, which were composed of both sericin protein and a small quantity of pigment. This implied that, at the equal mass concentration, the pigments have higher antioxidant activity than sericin. Although a number of recent studies as mentioned above have been conducted to identify the constituents of the silk pigment, the quantitative analysis of the silk pigments has not been well documented.

The objective of this study was therefore to investigate these high value pigments: carotenoids and flavonoids from silk waste of yellow Thai Nannoi silk. In this study, we first determined the total amount of carotenoids and flavonoids in silk waste sample. Then extraction of carotenoids and flavonoids from silk waste were conducted to determine the suitable conditions using two environmentally benign solvents, such as ethanol and subcritical water. Finally, the antioxidant activity of the extracts was determined.

1.2 Objectives

The objectives of this study are:

1.2.1 To determine the total amount of carotenoids and flavonoids extracted from Thai yellow silk (Nangnoi race) waste as well as the location of the pigments

1.2.2 To investigate the suitable environmentally benign solvents, to recover the pigments carotenoids and flavonoids, from Thai yellow silk (Nangnoi race) waste and determine the effects of important parameters, such as temperature and extraction time on the contents of the pigments.

1.2.3 To determine the antioxidant activity of the extracts obtained from Thai yellow silk waste.

1.3 Working scopes

1.3.1 To determine the total amount of carotenoids and flavonoids extraceted from silk waste from yellow silk, repeated batch ethanolic extraction was used.

1.3.2 To determine the location of carotenoids and flavonoids in silk waste.

1.3.3 To investigate the suitable environmentally benign solvents (ethanol and subcritical water) to recover the pigments: carotenoids and flavonoids.

1.3.4 To study the effect of temperature and reaction time on the content of pigments, 50-80°C and 2-12 hours were chosen as ethanolic extraction temperatures and reaction times, respectively. And for subcritical water extraction, we study on 120-150°C, 10-60 min.

1.3.5 To study an antioxidant activity of the extracts using ABTS radical cation scavenging assay.

1.4 Expected benefits

1.4.1 Recovery of valuable pigments which gives rise to the production of value added product from silk waste.

1.4.2 Removal of pigments from sericin and fibroin leads to the proteins that are colorless and thus are more attractive for some cosmeticeutical applications.

CHAPTER II

BACKGROUND AND LITERATURE REVIEWS

2.1 Introduction of silk

Silk is generally defined as spun fibrous protein polymer secretion produced by biological systems. It is synthesized by a variety of organisms including silkworms (and most other Lepidoptera lavae), spiders, scopions, mites and flies. Silkworm silk (*Bombyx mori* silk) has been the most intensively studied.

Figure 2.1 Silkworm, *Bombyx mori* [1]

[1]Source: http://perso.orange.fr/papillon.macro/bombyx.htm

| (a) | (b) | (c) |
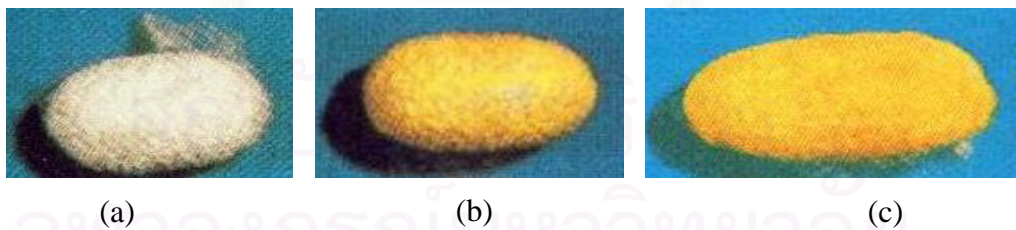
Figure 2.2 Silkworm (*Bombyx mori*) cocoon, (a) white Japanese race,

(b) yellow Spanish race, (c) yellow Japanese race[1]

[1]Source: Minakawa (1987)

The silkworm (Figure 2.1), which is actually a caterpillar, is the larva of the common, domesticated silkworm moth. Silkworms possess a pair of modified salivary glands called silk glands, which secrete a clear viscous fluid through spinnerets located on the mouthparts of the larva. This fluid hardens as it comes into contact with

air. The resultant silk fiber is used to spin a cocoon around the larva. Usually, one cocoon is made of a single thread about 300-900 meters long, and about 3,000-9,000 cocoons are needed to make a pound of silk, depending on the types of silkworm, which are typically classified into Thai, Japanese, Chinese or European. The pictures of cocoons of some types are presented in Figure 2.2.

2.2 Composition of silk fibre

Silk fibers consists of two main components which are the inner core protein, fibroin (70-80%), and the glue-like protein surrounding the silk fiber, sericin (20-30%) as shown in Figure 2.3. The other components of the fiber are hydrocarbon and pigment which constitute approximately 1.2-1.6 %wt. The compositions of silk may vary from species to species as can be seen in Table 2.1.
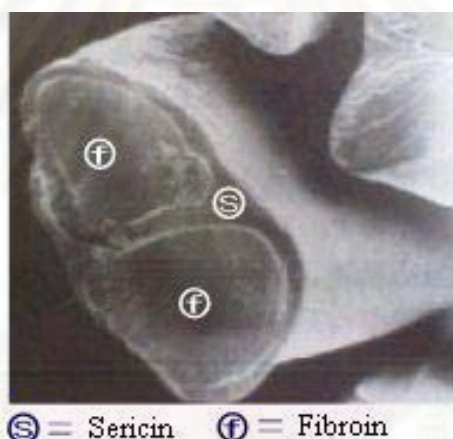
S = Sericin f = Fibroin

Figure 2.3 Structure of silk[1]

[1]Source: http://www.silk.or.jp/chuusan/kiito.htm

Table 2.1 Composition of silk from silk worm, *Bombyx mori*[1]

Species	Composition Position	% Fibroin	% Sericin	% water	% Alcohol	% Ether	%Carbon
N 122	Outer (30%)	65.86	31.36	10.69	1.44	1.36	0.893
X	Middle (64%)	77.3	20.97	10.24	1.03	0.7	0.904
C 122	Interior (6%)	73.57	23.78	10.06	0.96	1.69	0.922
Shuka	Outer (30%)	70.48	27.16	10.48	1.23	1.13	0.835
X	Middle (64%)	77.51	19.7	10.12	1.01	0.78	0.867
Girei	Interior (6%)	79.05	18.62	9.95	1.09	1.24	0.861
	Outer (30%)	73.25	24.13	10.37	1.42	1.2	0.804
Koishimaru	Middle (64%)	76.49	21.1	9.92	1.13	1.28	0.831
	Interior (6%)	77.55	20.05	9.93	1.01	1.39	0.858

[1]Source: Minagawa (1987)

2.3 Sericin

As mentioned earlier, the water soluble protein, sericin constitutes 20–30% of silk fiber. This glue like protein envelops the fibroin fiber with successive sticky layers that help in the formation of a cocoon. It ensures the cohesion of the cocoon by gluing silk threads together. Sericin is a complex mixture of 5–6 polypeptides widely differing in molecular weights, ranging from about 10 to over 300 kDa. It is made of 18 amino acids mainly, Serine (28.7%), Aspartic acid (17.9%), Glycine (9.1%), Threonine (7.9%) and Glutamic acid (7.9%). The compositions of amino acids in silk sericin and fibroin are shown in Table 2.2

Table 2.2 Amino acid composition of Fibroin and Sericin (g/ 100 g of protein)[1]

Amino acids	Fibroin	Sericin	Amino acids	Fibroin	Sericin
Glycine	42.8	8.8	Glutamic acid	1.7	10.1
Alanine	32.4	4.0	Serine	14.7	30.1
Leucine	0.7	0.9	Threonine	1.2	8.5
Isoleucine	0.9	0.6	Phenylalanine	1.2	0.6
Valine	3.0	3.1	Tyrosine	11.8	4.9
Arginine	0.9	4.2	Proline	0.6	0.5
Histidine	0.3	1.4	Methionine	0.2	0.1
Lysine	0.5	5.5	Tryptophan	0.5	0.5
Aspartic acid	1.9	16.8	Cystine	0.1	0.3

[1]Source: Kirimura (1972)

2.3.1 Application of sericin

Biodegradable materials

Sericin can be easily blended with other resin. Polyurethane foams incorporating sericin are said to have excellent moisture-absorbing and desorbing properties [Nomura et al., 1995]. The moisture absorption/desorption rates of the blended films are two to fivefold greater than that of the control film.

Membrane materials

Membranes are widely employed in reverse osmosis, dialysis, ultrafiltration and microfiltration. Pure sericin is generally not easily made into membranes, but

membranes of sericin cross-linked, blended, or copolymerized with other substances are made readily. Due to the fact that sericin contains a large amount of amino acids with neutral polar functional groups, sericin-containing membranes are quite hydrophilic and are permselective for water in an aqueous-organic liquid mixture such as water-ethanol [Mizoguchi et al., 1991].

Functional biomaterials

Sericin protein can be coated on surfaces of various durable materials to enhance functionality. The materials coated with sericin have excellent weatherability, good permeability, and do not warp on drying. Sericin blends well with water-soluble polymers, especially with polyvinyl alcohol (PVA). Ishikawa et al. (1987) investigated the fine structure and the physical properties of blended films made of PVA and sericin and found that, the blended film with 10–30% sericin in had good thermal and mechanical properties. By using simple technique, silk sericin could be coated onto nylon and polyester fibers and has a strong potential to be used for indoor air filters to reduce the amount of toxic free radicals, fungi, and micrococcus type of bacterias due to its anti-microbial activity [Sarovart et al., 2003].

Medical and cosmetic biomaterial

Sericin has been found to suppress lipid peroxidation and to inhibit tyrosinase (polyphenol oxidase) activity in vitro [Kato et al., 1998]. In cosmetic field, it has a unique affinity with other proteins which allows it to bind very effectively to the keratin of skin and hair, to form a multifunctional protective film resulting in a tightening, anti-wrinkle effect. The uniform film formed after the application of sericin results from its very high molecular weight. This substantive semiocclusive film persists even after washing, and protects the skin against harmful environmental influences. The sericin film leaves the skin with a smooth, silky feeling and substantiates several appropriate cosmetic effects.

Fuctional fibre fabric and articles

According to Yamada and Nomura (1998), sericin-coated fibers can prevent abrasive skin injuries and the development of rashes. In one study, synthetic fibers were coated by sericin by immersing in a 3% aqueous solution of sericin for a given time and then dried at 100 $^{\circ}$C for 3 min. The fabrics woven from the sericin-coated fibers were tested in products such as diapers, diaper liners, and wound dressing.

These sericin-coated fibers were good absorbent and did not cause skin rash. Also in this work, it was shown that rubber could be made more biocompatible by blending with sericin. A blend of hydrolyzed sericin (5–50 kDa molecular weight, 0.01–10.0% w/w) in rubber produces a product with reduced irritability to skin than native rubber. This modified rubber can be made into articles such as rubber gloves, bicycle handle grips, and handles for various sport equipment.

2.3.2 Silk sericin processing

For the preparation of silk fibre as a raw material for silk textile industries, removal of sericin layer is required to obtained purified fibroin fiber. The process is called degumming. Nowadays, many methods for degumming have been reported. The easiest one is by solubilizing of sericin in hot or boiling water. This can be done due to the hydrophilic properties of the sericin itself. However, this method is not favored as it usually gives incomplete degumming. Soap, synthetic detergent or alkali may be added to improve the degumming effect. For example, improved degumming could be achieved when 10-20 g/l soap is added, and the conditions are controlled at 92-98 $^{\circ}$C for 2-4 hours with the pH adjusted to 10.2-10.5. By using soap or alkali, the obtained silk must be washed with a large amount of water to rid of soapscum..

Therefore, a disadvantage of this method is that the use of a lot of water is required to thoroughly rinse the product. This causes a large amount of wastewater. Furthermore, the obtained silk is gloomy due to cation in soapscum.

Alternatively, many acids such as sulfuric, hydrochloric, citric acids, etc. can be used as a catalyst of the degumming process. But the use of these agents does not receive much attention in silk industry as the acid solutions are caustic and more toxic than the alkali solutions.

Similar to the hydrolysis of protein from any natural sources, sericin protein can be removed by using enzymes to accelerate the reaction. Freddi et al. (2003) reported that under optimum conditions of pH and temperature, the maximum amount of sericin removed in 1 h was 17.6 and 24 wt.% for alkaline proteases 3374-L (2 U/g fabric) and GC 897-H (1 U/g fabric), respectively, and 19 wt.% for a neutral protease, 3273-C (0.1 U/g fabric), while the use of an acid protease, EC 3.4 23.18, was almost ineffective as a degumming agent. In addition, the fabric obtained from

this method has a smooth skin. However, the cost of the enzymes makes this method unfavorable.

2.4 Fibroin

As shown in Table 2.2, fibroin is mostly composed of Glycine, Alanine, Serine and Tyrosine. Unlike sericin, fibroin is the principal water insoluble protein, that has a highly oriented and crystalline structure. Fibroin chains are generally aligned along the axis of silk fiber, by a close network of interchain hydrogen bonds, with adjacent -(ala–gly)$_n$- sequences forming the well known β-sheet crystals [Takahashi et al., 1991].

2.4.1 Application of fibroin

Membrane material

Fibroin can be used to make membranes for use in separation processes. For example, Hirotsu and Nakajima (1988) reported that an insolubilized silk fibroin membrane could be used to preferentially remove water from a mixture of water and alcohol.

Functional biomaterials

A blended hydrogel made of fibroin and PVA is said to have excellent moisture adsorbing and desorbing properties and elasticity [Yoshii et al., 2000]. Asakura et al. (1992) and Demura et al. (1992) reported coating glucose oxidase (GOD) on non-woven fibroin fabrics using sericin and/or fibroin aqueous solutions. When sericin alone was used for the GOD coating, a high activity of the immobilized enzyme was obtained but some leakage of sericin from the coated layer occurred. On the other hand, when GOD was coated on non-woven silk fibroin fabric using a mixture of sericin and fibroin and treated with 80% methanol, the insolubilization of the coated layer was markedly improved compared with the use of sericin alone.

Medical biomaterial

Tsubouchi (1999) developed a silk fibroin-based wound dressing that could accelerate healing and could be peeled off without damaging the newly formed skin.

The non-crystalline fibroin film of the wound dressing had a water content of 3–16% and a thickness of 10–100 mm. The silk proteins are also applicable as contact lens materials due to their high oxygen permeability, biocompatibility, and optical properties [Mori et al., 2000].

Cosmetics and Medication

A thin layer of silk powder adhered to the skin surface can absorb or release moisture along with the change of temperature and humidity of skin surface, to prevent and cure the dermatosis caused by moisture or dry conditions in certain degree, and to keep the skin in hygiene. Compared with talcum powder or titanium white powder, the silk powder have the excellent character of air permeability which can be applied to those powder beauty cosmetics, such as the facial powder or rouge, and etc. In addition, silk fibroin is an interesting polymer for drug delivery of polysaccharides and bioactive proteins due to the controllable level of crystallinity and the ability to process the biomaterial in biocompatible fashion under ambient conditions to avoid damage to labile compounds to be delivered [Hofmann et al., 2006].

Tissue Engineering

Due to the biocompatibility, slow degradability and remarkable mechanical properties of the material. Silk fibroin in various formats (films, fibers, nets, meshes, membranes, yarns, and sponges) has been shown to support stem cell adhesion, proliferation, and differentiation in vitro and promote tissue repair in vivo. In particular, stem cell-based tissue engineering using 3D silk fibroin scaffolds has expanded the use of silk-based biomaterials as promising scaffolds for engineering a range of skeletal tissues like bone, ligament, and cartilage, as well as connective tissues like skin [Meinel et al., 2004].

2.4.2 Silk fibroin processing

Prior to the regeneration of silk fibroin into a desirable form to meet a specific application, it must be converted into a solution form. It is known that silk fibroin is soluble in certain high ionic-strength aqueous solution, hence, conventionally, a fibroin solution can be obtained using various salt solution, such as aqueous lithium

thiocyanate (LiSCN), sodium thiocyanate (NaSCN), calcium thiocyanate (Ca(SCN)$_2$), calcium chloride (CaCl$_2$), lithium bromide (LiBr), and some copper salts. For example Yeo et al., 2002 reported the preparation of silk fibroin microsphere could be carried out by dissolving degummed silk fibroin in a mixture of CaCl$_2$:ethanol:H$_2$O = 1:2:8 in volume at 95 ^{o}C for 5 h. The silk fibroin solution was obtained after dissolved fibroin solution was dialyzed against distilled water for 4 days, from which fibroin powder could be obtained via freez drying. For the preparation of fibroin gel, Matsumoto et al. (2006) reported that the fibroin could be dissolved in 9.3 M LiBr solution at 60 °C for 4 h, yielding a 20 w/v % solution similar to the first example, this solution was dialyzed against distilled water for 2 days. The advantage of the method using these salt solutions is that large molecular weight was obtained, and therefore it is easy to form gel or film. However, a disadvantage of this method was found obviously that the impurities of toxic solvents associated, particularly when the protein is to be applied to biomedical and pharmaceutical products, thus they must be removed with dialyzation, a process which takes a long time of at least 2 days.

2.4.3 Subcritical water hydrolysis of silk sericin and fibroin

Subcritical water is water at near critical zone. The temperature ranges typically between the boiling and critical temperatures. Water is held in its liquid state above its normal atmospheric boiling point by the application of pressure. At such conditions, as shown in figure 2.4, water has high ion product (K$_w$). This means that the concentration of hydronium and hydroxide ions are increased and these ions would play an important role in breaking up large protein molecules into smaller proteins and amino acids through hydrolysis process. This results in the products that are soluble in water.

Recently, Lamoonphak et al., 2006 studied on the preparation of sericin and fibroin solution with subcritical hydrolysis method. The reaction was carried out in a closed batch reactor at 120-160 ^{o}C for the preparation of sericin solution, and at the higher temperatures of 160-220 ^{o}C for the preparation of fibroin solution. In this study, the effects of temperatures, reaction time (10-60 min), and silk to water ratios (1:20, 1:50, and 1:100) were examined on the amount of silk decomposed and on the proteins and amino acids yields.   The most suitable condition for protein and amino

(a)   (b)
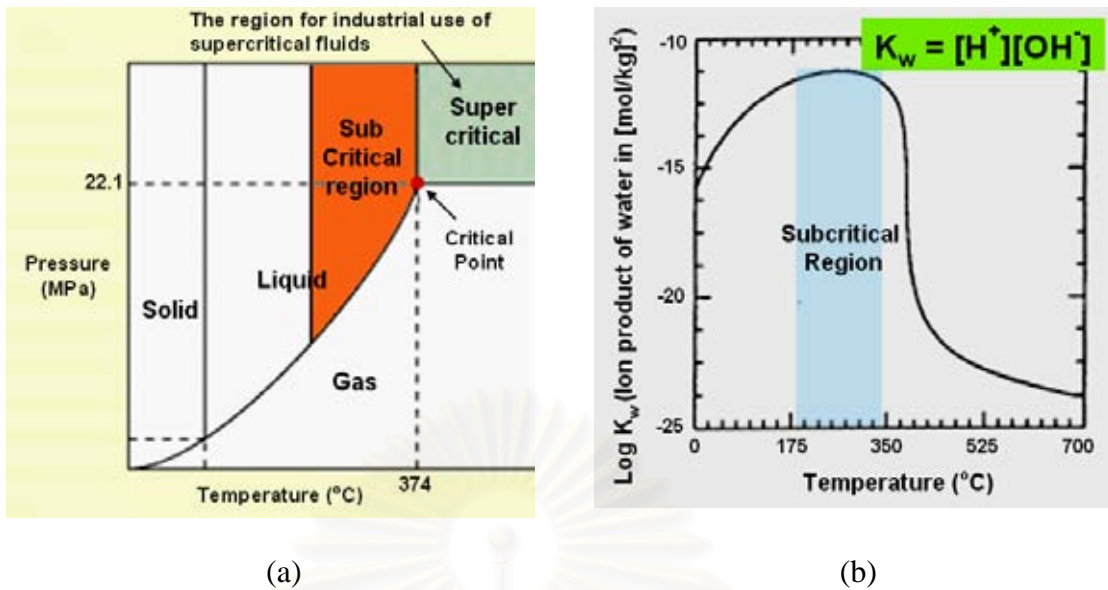
Figure 2.4 (a) Condition of subcritical water[1] and
(b) Ion product of water (K_w) at subcritical water region[1]

[1]Source: http://www.kobelco.co.jp/eng/p14/sfe01.htm

acids production from sericin by sub-critical water hydrolysis were 1:100 at 120 ^{o}C in 10 min (0.466 mg protein/mg raw silk) and 1:20 at 160 ^{o}C in 60 min (0.203 mg amino acids /mg raw silk), respectively. The most suitable condition for protein and amino acids production from fibroin by subcritical water hydrolysis were 1:100 at 220 ^{o}C in 10 min (0.455 mg protein/mg silk fibre) and 1:50 at 220 ^{o}C in 60 min (0.755 mg amino acids /mg silk fibre), respectively. However, this study did not report the effect of subcritical water conditions on antioxidant activity of the product, the activity that was reported not only to be a characteristics of silk sericin but that of the silk pigments, which are now known to be carotenoids and flavonoids [Tabunoki et al., 2004 and Tamura et al., 2002].

2.5 The pigments of silk

Silk cocoon colors of silkworm *Bombyx mori* vary, with naturally occurring shades of yellow, pink, golden-yellow, flesh, sasa (yellowish green) and green cocoons (figure 2.6). The yellow, pink, golden-yellow colors of the cocoons derived from carotenoids [Harizuka, 1953], while the colors of sasa and green silk cocoons are from flavonoids [Tamura et al., 2002 and Kurioka and Yamazaki, 2002]. These pigments are absorbed from mulberry leaves, transferred from the midgut to the silk

gland via hemolymph, and accumulated in the silk fiber
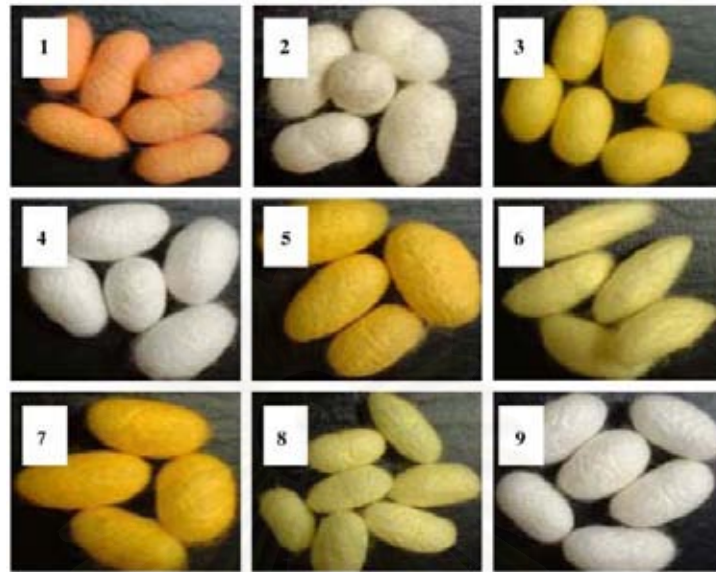
Figure 2.5 Races with different cocoon colors. (1) c440 (pink cocoon), (2)e21(whitish sasa), (3) N13 (yellow), (4) N15(white), (5) N16 (golden-yellow), (6) N17 (deep sasa), (7) N21 (golden-yellow), (8) N72 (pastel yellow) and (9) N71 (white)[1]

[1]Source: Tabunuki et al. (2004)

2.5.1 Carotenoids

Carotenoids are the most widespread group of natural pigments, with over 600 of carotenoids have been characterised structurally. They are present in all photosynthetic organisims and are components of the fruit, vegetables, and flowers having the yellow to red colors. Beside these sources, they are the characteristic colors of some birds, insects and marine invertebrates, which ingest carotenoids, in their diet and accumulate them in parts of their body tissues.

2.5.1.1 Structure and nomenclature

Carotenoids are isoprenoids generally consist of eight isoprene units joined together with covalent bond (Figure 2.6). The molecule of carotenoids could be presented in the form of straight chain such as lycopene, or may contain the ring at the tail of the chain like β-carotene. In general, carotenoids were classified into 2 groups,

hydrogenated and oxygenated carotenoids. Hydrogenated carotenoid derivatives or carotene group (Figure 2.7), such as β-carotene and lycopene, are the molecules that composed of hydrocarbon chain, which lead themselves to be characterized as non-polar molecules which can be easily dissolved in oil. The second group, called oxygenated carotenoid derivatives or xanthophylls group (figure 2.8) such as lutein, zeaxanthin, astaxanthin are the group of carotenoids whose molecules composed of oxygen atoms, thus they are more polar and dissolve less in oil than the carotenoids in the first group.

Figure 2.6 General formula of carotenoids,
broken lines indicate formal division into isoprenoid units[1]

[1]Source:http://www.chem.qmul.ac.uk/iupac/carot/car1t7.html
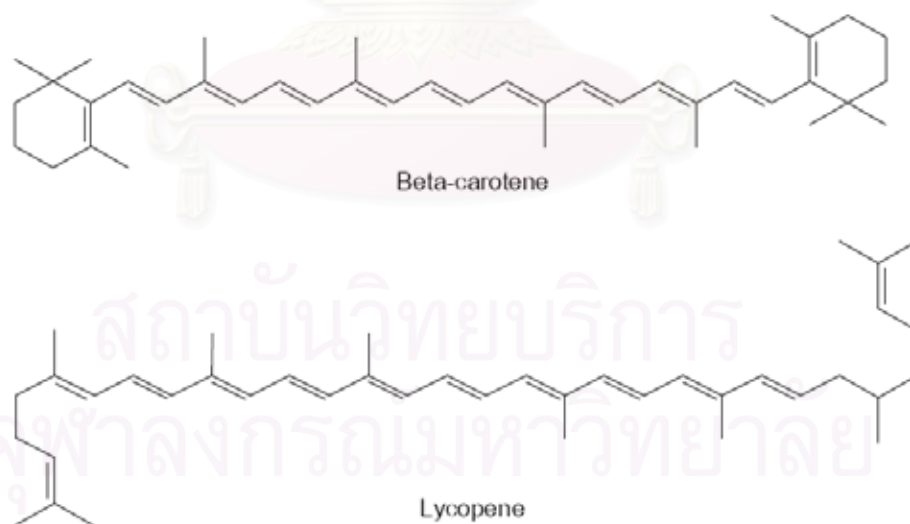
Figure 2.7 Chemical structure of hydrogenated carotenoids deriavatives[1]

[1]Source:Samee (2005)

Figure 2.8 Chemical structure of oxygenated carotenoids derivatives[1]

[1]Source:Samee (2005)

2.5.1.2 Dietary source of carotenoids

Carotenoids found in the human diet are primarily derived from crop plants, where the carotenoids are located in roots, leaves, shoots, seeds, fruit and flowers. Around 60 different carotenoids have been identified in fruits and vegetables consumed by humans [Khachik et al., 1992 and Scott et al., 1994]. Carotenoids can also be ingested in eggs, poultry and fish, where typically plant or algal products have been included in the feed of poultry or fish itself, e.g. zeaxanthin from maize in poultry feed. The typical amounts of carotenoids in crop plants are shown in Table 2.3.

2.5.1.3 Application of carotenoids

Carotenoids have a broad range of functions, especially in relation to human health. The role of carotenoids in the prevention of chronic diseases and their biological actions are summarized in figure 2.9.

Table 2.3 Carotenoid content of raw leafy green vegetables, fruits, roots and seeds[1]

Carotenoid content of raw leafy green vegetables, fruits, roots and seeds

Species	Carotenoid (mg/g fresh weight)					
	Total	Zea	Lutein	α-Carotene	β-Carotene	Lycopene
Brussel sprout	1163	-	610	-	553	-
Green bean	940	-	494	70	376	-
Broad bean	767	-	506	-	261	-
Broccoli	2533	-	1,614	-	919	-
Green cabbage	139	-	80	-	59	-
Lettuce	201	-	110	-	91	-
Parsley	10,335	-	5,812	-	4,523	-
Pea	2091	-	1,633	-	458	-
Spinach	9890	-	5,869	-	4,021	-
Watercress	16,632	-	10,713	-	5,919	-
Apricot	2196	31	101	37	1,766	-
Banana	126	4	33	50	39	-
Carrot (May)	11,427	-	170	2,660	8,597	-
Carrot (Sept)	14,693		283	3,610	10,800	-
Orange	211	50	64	Nd	14	-
Pepper	2784	1,608	503	167	416	-
Peach	309	42	78	Tr	103	-
Sweet corn	1978	437	522	60	59	-
Tomato	3454	-	78	-	439	2,937

Nd =not detected; Tr=trace

[1]Source:Scott et al. (1994)
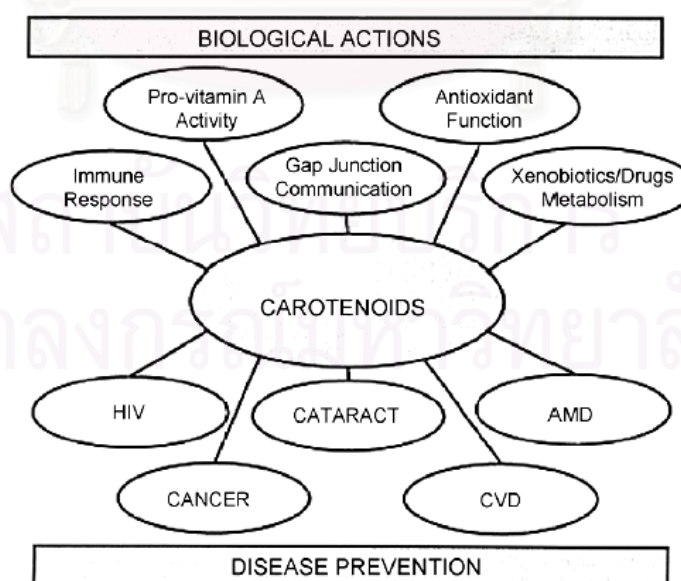
Figure 2.9 Role of carotenoids in the prevention of chronic diseases[1]

[1]Source:Rao and Rao (2007)

Provitamin A activity

The carotenoids composed of β-ring end groups, such as β-carotene, zeaxanthin and β-cryptoxanthin, when ingested in the diet, were reported to be cleaved by an enzyme, intestinal 15-15' –dioxygenase, at liver and intestine to form retinal or vitamin A which is important for human vision, sperm generating, bone and tooth generating, tissue restoration and skin nourishing.

Role in prevention of diseases

The first correlation between a high intake of carotenoids and its health benefits appeared in 1970s. Diets high in fruits and vegetables rich in carotenoids were associated with reduced rates of cancer and coronary heart disease. Many studies have speculated that the antioxidant activities of carotenoids are a key factor in reducing the incidence of many diseases.

Cancer

In vitro cell culture experiments have shown that carotenoids inhibit cell proliferation, transformation and micronucleus formation as well as modulating expression of certain genes. Among all carotenoids, lycopene was found to most efficiently inhibit the singlet state oxygen. This carotenoid is able to inhibit the oxidation of lipid on the membrane of DNA since it reacted with free radical. Several other studies since then demonstrated that with increased intake of lycopene and serum levels of lycopene the risk of cancers were reduced significantly [Rao and Agarwal, 1999 and Agarwal and Rao, 1998].

Cardiovascular disease (CVD)

Early epidemiological studies on the role of carotenoids in the development of CVD support the hypothesis that carotenoids have preventative potential [Kohlmier et al., 1995]. The consumption of processed tomato products does reduce lipoprotein sensitivity to oxidative damage [Hadley et al., 2003] and low serum lycopene is associated with an increased risk of atherosclerotic vascular events in middle-aged men [Rissanen et al., 2002].

Carotenoids and eyes health

The macular, an area within the retina in human eyes, contains two carotenoids: lutein and zeaxanthin. It is thought that these carotenoids play an important role in protecting the macula from light-induced damage, scavenge free radicals formed in the photoreceptors and protect eyes from some of the damaging effects of the sun by filtering blue light. Healthy lutein levels also help counteract the gradual deterioration of the macula from aging. Such damage to the macula can result in the onset of AMD, or age-related macular degeneration, the leading cause of vision loss [Hammond et al., 1997 and Wooten et al., 2002]. In addition, many researchers reported that people who take the dietary sources of high lutein and zeaxanthin such as spinach and broccoli are able to greatly reduce the risk of cataract [Landrum and Bone, 2001].

2.5.1.4 Carotenoids in silk

In many insects, absorption of dietary carotenoids is selective with a preference for carotenes in Orthoptera and Phasmida, and for xanthophylls (oxygenated carotenes) in Lepidoptera, as in the case for the silkworm, *Bombyx mori*. In *Bombyx mori*, the carotenoids are transported from the lumen of the midgut to the hemolymph lipoprotein, lipophorin, and from lipophorin into the silk gland, where the silk is produced. Once absorbed, carotenoids can be reversibly conjugated with proteins forming carotenoid-protein complexes called carotenoproteins or carotenoid-binding protein (CBP) which are water-soluble and more stable than the carotenoids alone [Chino et al., 1969, and Goodwin, 1953] In *Bombyx mori*, a major fate of the absorbed carotenoids is incorporated into the cocoon, which imparts various colors to the cocoon depending on the proportion of each type of carotenoid, lutein, β-carotene, α-carotene and other xanthophyll. For the yellow cocoon races, lutein is the main component of the yellow pigment which is contributed 88% of the carotenoids extracted from the CBP of race N4 (see figure 2.5) [Tabunoki et al., 2004]. The study of Jouini and Wells, 1996 informed that lutein is specifically and stoichiometrically bound to the protein, with a ratio of 3 mol of lutein per mol of protein.

2.5.1.5 Extraction of carotenoids

At present, many organic solvents have been used for extraction of carotenoids, such as that of the polar group such as acetone, methanol, isopropyl alcohol (IPA), ethyl acetate, ethanol, and etc, and that of the non-polar group such as hexane and petroleum ether. Sachindra et al. (2006) studied the suitable organic solvents for the recovery of carotenoids from shimp waste whose major pigment was astaxanthin xanthophylls. They found that the highest carotenoids yield (43.9 mg/g waste) from the shrimp waste was obtained when the carotenoids were extracted with a mixture of IPA and hexane. The yields obtained using various organic solvents are summarized in Table 2.4

Table 2.4 Yield of carotenoids from shrimp waste in different solvents and solvent mixtures[1]

Solvent/solvent mixture	Yield (μg/g waste) (WWB)*
Acetone	40.6 ± 1.6
Methanol	29.0 ± 3.3
Ethyl methyl ketone	36.8 ± 1.9
Isopropyl alcohol (IPA)	4.1 ± 3.0
Ethyl acetate	36.9 ± 2.9
Ethanol	31.9 ± 2.2
Petroleum ether	12.1 ± 1.8
Hexane	1.3 ± 1.9
Acetone:hexane (50:50)	38.5 ± 1.0
IPA:hexane (50:50)	43.9 ± 0.7

* WWB – wet weight basis

[1]Source: Sachindra et al. (2006)

In addition, Delgado-Vargus et al., 2000 discussed the advantages and the disadvantages of various organic solvent for extraction of carotenoids and suggested that polar solvent are generally good extraction media for xanthophylls but not for carotenes due to the reason associated with their hydrocarbon structure which is

known as a non-polar form.

2.5.2 Flavonoids

Flavonoids constitute a group of phenoilic substances found in many terrestrial or floating plants, but not aquatic plants. The compounds can efficiently adsorb an ultraviolet radiation, and then release it as diverse colors, and thus they are said to contribute to the colors of the plants (fruits or petals). Through evolution, the plants are believed to have produced flavonoids to protect themselves from ultravilolet radiation.

2.5.2.1 General Flavonoids Structure

The main structure of flavonoids comprises of 15 carbons, arranged in the form of two benzene rings (A and B), which are connected by an oxygen-containing pyrene ring (C) (Figure 2.10). Flavonoids containing a hydroxyl group in position C-3 of the ring are classified as 3-hydroxyflavonoids (flavonols, anthocyanidins, leucoanthocyanidins, and catechins), and those lacking it as 3-desoxyflavonoids (flavanones and flavones). Classification within the 2 families is based on whether and how additional hydroxyl or methyl groups have been introduced to the different positions of the molecule. Isoflavonoids differ from the other groups; the B ring is bound to C-3 of ring C instead of C-2. Anthocyanidins and catechins, on the other hand, lack the carbonyl group on C-4. Different derivatives of the most common flavonoids are summarized in Table 2.5.

Generally, flavonoids in plants are present mainly as glycosides. Aglycones (the forms lacking sugar moieties) occur less frequently. At least 8 different monosaccharides or combinations of these (di- or trisaccharides) can bind to the different hydroxyl groups of the flavonoids aglyone. The large number of flavonoids
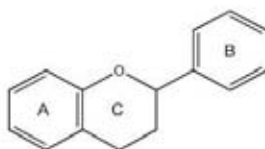
Figure 2.10 General Flavonoids Structure[1]

[1]Source: http://lpi.oregonstate.edu/infocenter/phytochemicals/flavonoids/basicflav.html

Table 2.5 Nomenclature of the subclass of flavonoids based on the position of their substituents[1]

	3	5	7	2'	3'	4'	5'
Flavonols:							
Kaempferol	OH	OH	OH	H	H	OH	H
Morin	OH	OH	OH	OH	H	OH	H
Rutin	O-R1	OH	OH	H	OH	OH	H
Myricetin	OH	OH	OH	H	OH	OH	OH
Quercetin	OH	OH	OH	H	OH	OH	H
Quercetrin	O-Rh	OH	OH	H	OH	OH	H
Myricitrin	O-Rh	OH	OH	H	OH	OH	OH
Spirenoside	OH	OH	OH	H	OH	O-Glu	H
Galangin	OH	OH	OH	H	H	H	H
Robinin	O-R1	OH	OH	H	H	OH	H
Kaempferide	OH	OH	OH	H	H	O-Me	H
Fisetin	OH	H	OH	H	OH	OH	H
Rhamnetin	OH	OH	O-Me	H	OH	OH	H
Flavonones:							
Hesperitin	H	OH	OH	H	OH	O-Me	H
Naringin	H	OH	O-R	H	H	OH	H
Naringenin	H	OH	OH	H	H	OH	H
Eriodictyol	H	OH	OH	H	OH	OH	H
Hesperidin	H	OH	O-Me	H	OH	O-Me	H
Pinocembrin	H	OH	OH	H	H	H	H
Likvirtin	H	H	OH	H	H	O-Glu H	H
Flavones:							
Rpoifolin	H	OH	O-R	H	H	OH	H
Apigenin	H	OH	OH	H	H	OH	H
Tangeretin	H	O-Me	O-Me	H	H	O-Me	H
Flavone	H	H	H	H	H	H	H
Baicalein	H	OH	OH	H	H	H	H
Luteolin	H	OH	OH	H	OH	OH	H
Chrysin	H	OH	OH	H	H	H	H
Techtochrysin	H	OH	O-Me	H	H	H	H
Diosmetin	H	OH	OH	H	OH	O-Me	H
Diosmin	H	OH	O-R1	H	OH	O-Me	H
Flavanolols:							
Silibinin	OH	OH	OH	H	H	O-L-O-	H
Silymarin	OH	OH	OH	H	H	O-L-O-	H
Taxifolin	OH	OH	OH	H	OH	OH	H
Pinobanksin	OH	OH	OH	H	H	H	H
Flavan-3-o1s:							
Catechin	OH	OH	OH	H	OH	OH	H
Isoflavones:							
Genistein	-	OH	OH	H	H	OH	H
Daidzin	-	H	O-Glu	H	H	OH	H

-O-Me = Methoxy   -O-Glu = Glucosyl   -O-J31 = Alkoxy   -O-L-O = Selane

[1]Source: Raj narayana et al. (2001)

is a result of the many different combinations of flavonoids aglycones and these sugars. The most common sugar moieties include D-glucose and r-rhamnose. The glycosides are usually O-glycosides, with the sugar moiety bound to the hydroxyl group at the C-3 or C-7 position. Some dietary sources of flavonoids are summarized in table 2.6

Table 2.6 Some Dietary Sources of Flavonoinds[1]

Flavonol	
Quercetin	onion, lettuce, broccoli, cranberry, apple skin, berries, olive, tea, red wine
Kaempferol	endive, leek, broccoli, radish, grapefruit, black tea
Myricetin	cranberry, grapes, red wine
Flavanone	
Naringin	peel of citrus fruits
Taxifolin	citrus fruits
Flavone	
Chrysin	fruit skin
Apigenin	celery, parsley
Flavanol	
Catechin	red wine
Anthocyanidins	
Malvidin	red grapes, red wine
Cyanidin	cherry, raspberry, strawberry, grapes
Apigenidin	coloured fruit and peels
Isoflavonoid	
Genistein	Soybeans

[1]Source: Rice-Evans et al. (1995)

2.5.2.2 Application of Flavonoids

Flavonoids of different classes have several pharmacological activities.

Cardiotonic activity

Flavonoids have been reported to have action on the heart In recent report the cardiotoxicity (negative inotropic effect) of doxorubicin on the mouse left atrium has been inhibited by flavonoids, 7-monohydroxy ethyl rutoside and 71,3',4'-trihydroxyethyl rutoside. The glycosides of luteolin, apigenin and genistein produced antihypertensive activity even more than the reference drug papaverine. Three flavonoids showed vasorelaxant effect in order of potency, luteolin > eriodictyol >

naringenin on rat thoracic aorta. Different flavonoids were tested for a positive inotropic effect on guinea pig papillary muscle placed at 0.2 Hz in a Kerbs-Henseleit solution at 30° C. Quercetin showed the most potent intrinsic activity, and produced the strongest inotropic responses among the different flavonoids. The relative order of potency of the tested flavonoids was, quercetin > morin = kaempferol > luteolin = apigenin> fisetin = galangin [Itoigawa et al., 1999].

Antioxidant activity

Antioxidants are compounds that protect cells against the damaging effects of reactive oxygen species, such as singlet oxygen, superoxide, peroxyl radicals, hydroxyl radicals and peroxynitrite. An imbalance between antioxidants and reactive oxygen species results in oxidative stress, leading to cellular damage. Oxidative stress has been linked to cancer, aging, atherosclerosis, ischemic injury, inflammation and neurodegenerative diseases (Parkinson's and Alzheimer's).

The recognized dietary antioxidants are vitamin C, vitamin E, selenium, and carotenoids. Like alpha-tocopherol (vitamin E), flavonoids contain chemical structural elements that may be responsible for their antioxidant activities. It was suggested that flavonoids can replace vitamin E as chain-breaking anti-oxidants in liver microsomal membranes. The contribution of flavonoids to the antioxidant defense system may be substantial considering that the total daily intake of flavonoids can range from 50 to 800 mg. This intake is high compared to the average daily intake of other dietary antioxidants like vitamin C (70 mg), vitamin E (7-10 mg) or carotenoids (2-3 mg). Flavonoids intake depends upon the consumption of fruits, vegetables, and certain beverages, such as red wine, tea, and beer. The high consumption of tea and wine may be most influential on total flavonoids intake in certain groups of people. The capacity of flavonoids to act as antioxidants depends upon their molecular structure. The scavenging activity of flavonoids has been reported to be in the order: myricetin > quercetin > rhamnetin > morin > diosmetin > naringenin > apigenin > catechin > 5,7-dihydroxy-3',4',5'-trimethoxyflavone > robinin > kaempferol > flavone [Ratty et al., 1988]. They have also been suggested to play a protective role in liver diseases, cataracts, and cardiovascular diseases. Quercetin and silybin acting as free radical scavengers were shown to exert a protective effect in reperfusion ischemic tissue damage [Hillwell et al., 1994]. Stabilization of meat lipids with flavonoids has been studied. Induction period of lipid oxidation in canola oil was delayed with the

flavonoids myricetin by up to fifteen days. Formation of oxidation products was also inhibited by 69%, during this period. Morin, myricetin, kaempferol and quercetin have also been suggested as stabilizers for fish oil as an alternative to synthetic antioxidants [Husain et al., 1987].

Anti-inflammatory activity

A number of flavonoids are reported to possess anti-inflammatory activity. Hesperidin, a citrus flavonoid possesses significant anti-inflammatory and analgesic effects [Shahidi et al., 1998]. Recently, apigenin, luteolin and quercetin have been reported to exhibit anti-inflammatory activity. Quercetin, gallic acid ethyl ester, and some as yet unidentified flavonoids might account for the antinociceptive action reported for the hydroalcoholic extract of Phyllanthus caroliniensis.

Effects on blood vessels

Quercetin and rutin have been used as effective constituents of several pharmaceuticals used for treatment of capillary fragility and phlebosclerosis. The activities of certain flavonoids in inhibiting capillary permeability. It has been suggested that flavonoids, which contain free hydroxyl groups at 3, 3' and 4' positions exert beneficial physiological effects on capillaries. Flavonoids tangeratin, hesperidin, quercetin, and rutin have been found to reduce aggregation of horse erythrocytes. Nobeletin and sinensetin decreased erythrocyte aggregation and sedimentation in vitro and might be useful in dietary control of high blood viscosity syndrome [Fritz et al., 1996].

Antimicrobial activity

Flavonoids and esters of phenolic acids were investigated for their antibacterial, antifungal and antiviral activities.

Antibacterial Activity: Twenty-five out of one hundred and eighty two flavonoids studies were found to be active against many bacteria. Most of the flavonones having no sugar moiety showed antimicrobial activities [Wild et al., 1969]

Antifungal Activity: Number of flavonoids isolatedfrom peel of tangerine orange, when tested for fungistatic activity towards Deuterophoma tracheiphila showed promising activity [Tencate et al., 1973]

Antiviral Activity: Flavonoids also displayed antiviral, including anti-HIV

activity. It has been found that flavonols are more active than flavones against herpes simplex virus type 1 and the order of importance was galangin > kaempferol > quercetin [Thomas et al.,1988].

2.5.2.3 Flavonoids in silk

The color components of the silk cocoon shell are associated with flavonoids phenolic compounds whose components varies depending on the *B. mori* strain. Kurioka and Yamazaki (2002) identified flavonoids from the yellow green cocoon shells of the silkworm, *Bombyx mori*, by examining the ethanolic extracts of the cocoon shells with HPLC-ESI-MS. They found that quercetin 7-o-β-D-glucoside (34.8%) and kaempferol 7-o-β-D-glucoside (27.5%) constitute the maximum quantity of total flavonoids in the cocoon shells. The others are 17.5% quercetin 5-o-β-D-glucoside, 15.3% quercetin 4'-o-β-D-glucoside, 13.4% quercetin, 7.4% kaempferol 5-o-β-D-glucoside and 1.2% kaemferol.

In other research of Tamura et al. (2002), the flavonoid 5-glucosides, quercetin 5,4'-di-O-β-D-glucopyranoside and quercetin 5,7,4'-tri-O-β-D-glucopyranoside, together with the known quercetin 5-O-β-D-glucopyranoside were isolated from the cocoon shell of the silkworm, *Bombyx mori*. The structures were identified by spectroscopic analysis. These flavonoid glucosides were not present in mulberry leaves, and therefore they are considered to be metabolites produced by the silkworm.

Our preliminary experiment, the total flavonoids content of ethanolic extract (80 $^{\circ}$C, 3 hour with 1:50 of silk to aqueous ratio) was determined according to the aluminum chloride colorimetric method described by Chang et al. (2002). The flavonoids content from yellow silk waste was 170.32 mg/100 g dry weight, which is more than that of some fruits and vegetables such as dry outer skin of onion (96 mg/100 g of dry material), spring onion leaves (45 mg/100 g of dry material), apple peel (21 mg/100 g of dry material), etc. [Wach et al., 2005].

Thongphasuk et al. (2005) examined antioxidant activity and tyrosinase inhibitory activity of ethanolic extracts and water extracts of three races cocoons: Nangnoi (yellow cocoons), UB1 (white cocoons) and Lao (cream cocoons) as well as the extracts of mulberry (Morus alba L.) leaves. The results showed that, for the same raw material, the antioxidant activity and tyrosinase inhibitory activity in the ethanolic

extracts were higher than that of water extracts. The ethanolic extracts of four kinds of raw materials showed antioxidant effect in the following order of potency; Nangnoi > Lao > Mulberry leaves > UB1 and for tyrosinase inhibitory effect, Nannoi > UB1 > Mulberry leaves > Lao. These results implied that antioxidants and tyrosinase inhibitory effects found in the water extract of silk cocoons are not only resulted from water soluble protein sericin, but also from flavonoids contained within the extracts. With the equal concentrations, flavonoids might have higher potencies of antioxidant activity and tyrosinase inhibitory activity than that of sericin.

2.5.2.4 Extraction of Flavonoids

The extraction using alcohols as a solvent is a conventional method to obtain many flavonoids from various natural sources. Among the alcohols, ethanol is the most favored due to the fact that it acts as an environmentally friendly and safe for food use. Xu et al., 2005 studied the effect of various parameters such as temperatures, extraction times, concentrations of alcohol and material:aqueous ratios for ethanolic extraction of flavonoids from red-raspberry. They found extraction temperature contributed to the increased the yield of total flavonoids, up to the temperature of 80 $^{\circ}$C, in which the reduction of flavonoids yield was found. Longer extraction time, also increased the yield, especially, during the first 2 to 3 hours of extraction, in which the exponential increasing of flavonoids yield was found. However the yield decreased after longer extraction time. A small amount of EtOH aqueous solution (1:10 w/v sample to solvent ratio) is sufficient to reach the highest yield. For the effect of alcohol (EtOH) concentration in water, 75% EtOH solution was found to be adequate.

2.6 Antioxidant activity

To study on antioxidant activity, the definition of "free radical" are firstly need to clarified. Free radicals are highly unstable and reactive molecules that attack, infiltrate and injure vital cell structures. They are constantly formed as a natural by-product of body chemistry. Free radicals are highly toxic and can damage important cellular molecules such as DNA, lipids or other parts of the cell, impairing the brain and other tissue. They are believed to accelerate the progression of age-related

conditions and other diseases.

The main characteristic of an antioxidant activity is its ability to trap free radicals. Therefore, antioxidant compounds in food play an important role as a health-protecting factor. Scientific evidence suggests that antioxidants reduce the risk for chronic diseases including cancer and hearth disease. Plant sourced food antioxidants like vitamin C, vitamin E, carotene, phenolic acids, phytate and phytoestrogens have been recognized as having the potential to reduce diseases risk.

2.6.1 Monitoring of antioxidant activity

Various antioxidant activity methods have been used to monitor and compare the antioxidant activity of foods. These analytical methods measure the radical-scavenging activity of the antioxidants against free radicals like 2,2-azinobis (3-ethyl-benzothiazoline-6-sulfonic acid) (ABTS) [Leong and Shui, 2002, Miller and Rice-Evans, 1997], 2,2-diphenyl-1-picrylhydrazyl (DPPH) [Brand-Williams et al.,1995, Gil et al., 2002], ferric reducing antioxidant power (FRAP) [Benzie and Strain, 1999, Guo et al., 2003], and the oxygen radical absorption capacity (ORAC) [Cao et al., 1993]. Among all of these assays, ABTS and DPPH are widely used.

ABTS

ABTS radical cation decolorization assay is a rapid and reliable method that is widely used in the total radicalscavenging measurement of pure substances, aqueous mixtures, and beverages. The reaction between ABTS and potassium persulfate directly generates the blue/green ABTS chromophore, which can be reduced by an antioxidant, thereby resulting in a loss of absorbance at 734 nm.

DPPH

DPPH is a stable free radical and is widely used to assess the radical-scavenging activity of antioxidant compounds. This method is based on the reduction of DPPH in methanol solution in the presence of a hydrogen-donating antioxidant due to the formation of the non radical form DPPH-H. This transformation results in a color change from purple to yellow, which is measured spectrophotometrically. The disappearance of the purple color is monitored at 517 nm.

Antioxidant activity of some medicinal plants and some tropical fruits were measured using DPPH assay and summarized in table 2.7 and 2.8, respectively

Table 2.7 The antioxidant activities of extracts from medicinal plants, measure by DPPH assay[1]

Plant	Part used	IC_{50} (µg/ml)
Copernicia cerifera	Leaves	23.5 ± 0.1
	Mesocarp of fruits	15.3 ± 0.4
	Epicarp of fruits	41.9 ± 0.8
Mauritia vinifera	Mesocarp of fruits	538.3 ± 2.0
	Epicarp of fruits	71.0 ± 1.0
Syagrus oleracea	Epicarp of fruits	425.5 ± 1.9
	Epicarp/mesocarp of fruits	27.0 ± 0.2
Orbigynia speciosa	Endocarp of fruits	4104.3 ± 6.7
	Flowers	427.4 ± 1.8
	Leaves	895.9 ± 2.3
Bauhinia variegata	Aerial parts	37.0 ± 0.4
Bauhinia purpurea	Aerial parts	137.9 ± 1.1
Bauhinia candida	Aerial parts	45.4 ± 0.9
Bauhinia monandra	Aerial parts	199.8 ± 1.6
Bauhinia angulosa	Aerial parts	106.4 ± 1.1
	Aerial parts	$68.4 \ 1.0$
	Aerial parts	$35.1 \ 0.3$
Polygala paniculata	Aerial parts	135.4 ± 1.1
	Roots	325.6 ± 1.6
Hyptis fasciculata	Aerial parts	57.9 ± 0.9
	Aerial parts	35.0 ± 0.3
Hyptis heterodon	Aerial parts	233.4 ± 1.3
Ginkgo biloba	Leaves	41.5 ± 0.1

[1]Source: Silva et al. (2005)

Table 2.8 The antioxidant activities of extracts from tropical fruits, measure by DPPH
assay [1]

Fruits	IC$_{50}$ (mg/ml) [a]
Guava (seeded)	1.71 ± 0.61
Guava (seedless)	2.11 ± 0.63
Banana (mas)	13.4 ± 2.5
Gragon fruit	27.5 ± 3.9
Star friut	3.8 ± 2.1
Sugar apple (brown)	3.9 ± 0.4
Sugar apple (green)	4.6 ± 0.8
Water apple	12.0 ± 3.8
Orange	5.4 ± 1.3

[a]The IC$_{50}$ values (the concentration required to inhibit radical formation by 50%)

[1]Source:Lim et al. (2006)

CHAPTER III

MATERIALS AND METHODS

3.1 Materials

Silk waste is obtained from yellow cocoon (Nangnoi race) of silkworm, *Bombyx mori* from Queen Sirikit Sericulture Centre, Tak, Thailand. All chemicals used for the determination of total flavonoids, quercetin dehydrate, aluminum chloride hexahydrate, and potassium acetate, and those used for the determination of total carotenoids, lutein and β-carotene are analytical grade were purchased from Sigma-Aldrich, Germany. Ethanol used as a solvent for extraction of the pigments was also of analytical grade, and was purchased from Merck, USA.

3.2. Preparation of silk waste raw material

Silk waste was cleaned by first removing the contaminants manually and the silk was then washed twice with water. The cleaned silk waste was then dried in vacuum oven at 60 ^{o}C for about 24 hours. Prior to extraction of the pigments, the silk waste was cut into short fragments whose average length was 100 mm.

3.3 Determination of the total amount of carotenoids and flavonoids in Thai yellow silk waste

In order to determine the total amount of carotenoids and flavonoids within the silk waste sample, the pigments were removed from the yellow silk sample by repeated extraction in a 100 ml batch pressure vessel (SUS- 316 stainless steel, AKICO, Japan). The system is schematically shown in Figure 3.1 In this system, three grams of the silk waste sample was extracted with 60 ml of 95% (v/v) ethanol at 80°C first for 2 h, after which the vessel was immediately cooled to room temperature by submerging it into a water bath. The remaining silk fibre was separated from the soluble product using a filter paper (Watman no. 1, Maidstone, England). The residue silk was again charged into the reactor with the same solvent system for the repeated

extraction. The repeated extractions were 5 times carried out following table 3.1 The extracts was then determined for the amount of total carotenoids and flavonoids by a spectrophotometer.
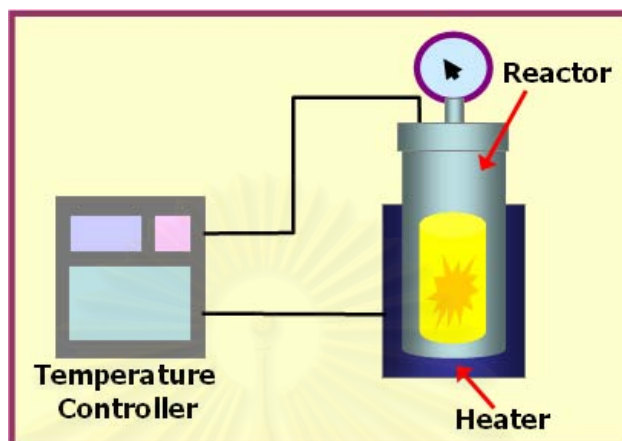
Figure 3.1 Schematic diagram of batch system for subcritical water hydrolysis

Table 3.1 Repeated condition for the recovery of carotenoids and flavonoids

Repeated Time	Temperature (^{o}C)	Extraction Time (h)
1		2
2		2
3	80	3
4		3
5		3

3.4 Determination of the distribution of carotenoids and flavonoids in silk waste

To determine the distribution of flavonoids in the silk samples (sericin versus fibrion), extraction was carried out first to completely remove sericin by subcritical water at 150°C for 30 min in the pressure vessel in Figure 3.1. This process has been demonstrated to remove the sericin protein completely from the silk waste sample [Lamoolphak et al., 2006]. The remaining fibroin fiber was then extracted repeatedly in a batch pressure vessel with ethanol following the method previously described

until the pigments were completely removed. The contents of both carotnoids and flavonoids in fibroin protein were then analyzed by a spectrophotometer and the amount of pigments on the sericin was the difference between the total amount and that of the pigments in the fibroin. For carotenoids, this is a suitable approximation as only a small amount of carotenoids could be extracted in to the subcritical water. For flavonoids, it could be possible that some amount of flavonoids from fibroin might be extracted with subcritical water. Nevertheless, it was difficult to determine the exact amount of the compound on fibroin and sericin as physical separation of sericin and fibroin layer could not easily achieved.

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3.5 Determination of suitable extraction solvents and conditions

3.5.1 Subcritical water extraction

To determine the suitability of using subcritical water for extraction of flavonoids and carotenoids from silk waste, the experiment was carried out in a batch pressure vessel described previously. The extraction procedure was similar to that used for sericin extraction described by Lamoolphak et al. (2006). Firstly, silk waste and water at the mass ratio of 1:50 was charged into the reaction vessel. The vessel was then tightened and heated to the desired temperature (120-150°C) and reaction time (10-60 min) to determine the variable effects on the total amount of pigment extracted and antioxidant activity. After a specified extraction time was reached, the reactor was immediately cooled to room temperature by submerging it into a water bath. The remaining silk fibre was separated from the soluble product using a filter paper (Watman no. 1, Maidstone, England). The residue was then dried in a vacuum oven at 60°C for about 6-8 h, and dried weight was measured. The solution was then assayed to determine the amount of total carotenoids and flavonoids and their antioxidant activity.

3.5.2 Ethanolic extraction

The effects of extraction temperature (50-79°C) and the extraction time (2-12 h) were studied for extraction of silk pigments using a heat reflux extraction system. Two grams of yellow silk waste and 100 ml of 95% ethanol were charged into a round

bottom flask, which was then heated to desire temperatures. The extraction was carried out for the total of 12 h during which 2 ml of sample was taken in every 2 h interval, and replenished with the same volume fresh solvent. The samples were analyzed both for the amount of total carotenoids, flavonoids, and the antioxidant activity of the extracts.

3.6 Determination of total carotenoids and flavonoids content

The total carotenoids content in the extracts was determined by measuring the absorbance at 478 nm with a spectrophotometer using lutein as a standard. The total flavonoid content was determined using the aluminum chloride colorimetric method [Chang et al., 2002]. Briefly, as a standard, quercetin solutions were prepared at 12.5, 25.0, 50.0, 80 and 100 mg/ml in 80% Ethanol (V/V). For the analysis, 0.5 ml of the extracted samples or quercetin solutions were mixed with 1.5 ml of 95% Ethanol (V/V), 0.1 ml of 10% aluminum chloride (m/V), 0.1 ml of 1 mol/L of potassium acetate, and 2.8 ml of distilled water, and the mixture was incubated at room temperature for 30 minutes. The absorbance of the mixture was then measured by a spectrophotometer at 415 nm. For the blank sample, 10% (m/V) aluminum chloride in the mixture solution was substituted by the same volume of distilled water.

3.7 Determination of antioxidant activity

ABTS (2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) radical cation scavenging assay was carried out following a modified method described by Re et al. (1999). The extract was diluted in series in water and each diluted solutions were added into $ABTS^{\bullet+}$ solution (aqueous solution of 7mM ABTS and 2.45 mM potassium persulfate having absorbance of 0.70 ± 0.02 at 734 nm) with the volume ratio of 1:30 (sample solution:ABTS solution). The solutions were mixed using a vortex and the mixtures were incubated in the dark at room temperature for 10 min, after which the absorbance was measured at 734 nm.

For comparing the antioxidant activity of the extracts obtained at various conditions, concentration of sample producing 50% reduction of the radical absorbance (IC_{50}) was used as an index. The IC_{50} values for various extracts were

found from the plots of percent inhibition (PI) versus the corresponding concentration of the sample. The values of PI were calculated using the following equation:

$$PI\ (\%) = [1-(A_t\ /A_r)] \times 100$$

where A_t and A_r are absorbance of test sample and absorbance of the reference, respectively.

3.8 HPLC analysis

The carotenoid constituents were determined by HPLC following the method of Heinonen (1990). The sample extract was injected to the Lichrocart C-18 column, isocratic separation of carotenoids was accomplished, with a mobile phase consisting of acetonitrile: dichloromethane: methanol (70:20:10 (v/v)), at a flow rate of 1.5 ml/min and the column was maintained at room temperature. The effluent was monitored at 450 nm.

The flavonoid constituents of the extracts were also determined using HPLC by modified from the method of Cai et al. (2003). HPLC were performed with a C-18 Inertsil ODS-3 column (5 µm particle, 4.6 ×250 mm ID) and equipped with UV detector. The UV detector absorbance was monitored at 254 nm. The mobile phases consisted of solvent A (0.1% trifluoroacetic acid in acetonitrile), solvent B (0.1% trifluoroacetic acid in HPLC grade water), and solvent C (100% methanol, HPLC grade). Flow rate was set at 1.0 ml/min, and column temperature was maintained at 37 °C throughout of the test. The initial solvent composition was 0% solvent A and 100% solvent B. A linear gradient was used to increase solvent A from 0% to 10% within 7 minutes. This solvent composition was maintained at an isocratic flow for 3 min. The solvent A was then increased from 10% to 40% using a 20-min linear gradient. This composition was maintained for 2 min and returned to the initial composition in 3 min. Solvent C was used for washing the column after each run.

CHAPTER IV

RESULTS AND DISCUSSIONS

4.1 Total amount of flavonoids and carotenoids in silk waste

In order to determine the total carotenoid and flavonoid contents in the waste of Thai yellow Nangnoi silk, the repeated extraction of the silk waste was conducted with ethanol at 80°C for 5 times, each for the period of 2-3 h. The final extract appeared to be clear solution, suggesting that the yellow color of the raw material was completely removed as shown in Figures 4.1 and 4.2. The contents of total

Figure 4.1 Ethanolic extracts of 5 times of repeated extraction of the silk waste
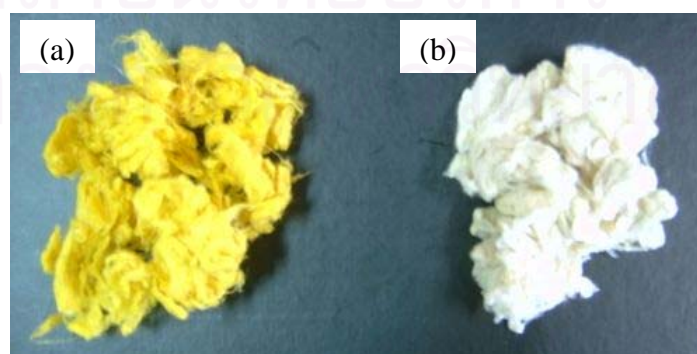
Figure 4.2 Thai yellow silk waste,

(a) raw silk, (b) after 5 times of repeated extraction of the silk waste

carotenoids and flavonoids were quantified for all the samples which are summarized in table 4.1 and 4.2, respectively. The result of table 4.1 shows that the silk waste contains the total carotenoids content of 0.73 mg/g of dry weight as determined by spectrophotometric method. For HPLC analysis, it was found that the repeated extracts contain lutein as major (Figures 4.3 and 4.4). This compound was eluted at the retention time of 1.5 min. Quantitative analysis of lutein (see figure A-3 in appendix A) showed that this xanthophyll was accounted for 0.64 mg/g of dry silk. By comparison with other sources, this lutein content in the silk waste appears to be in the same range as many vegetables which are reported as important sources of lutein such as kale (0.6-0.8 mg/g dry weight) [Lefsrud et al., 2007] and spinach (0.6-0.7 mg/g dry weight) [Kopsell and Lefsrud, 2006].

Table 4.1 The content of total carotenoids in Thai yellow silk waste, Nangnoi race

No. of repeated batch	Abs 478 nm	Conc. of total carotenoids (mg/ml)[c]	Content of total carotenoids (mg/g dry weight)[d]	% of total carotenoids
1[a]	4.2590	0.0238	0.476	65.44
2[a]	1.6625	0.0093	0.186	25.54
3[b]	0.4030	0.0023	0.045	6.19
4[b]	0.1740	0.0010	0.019	2.67
5[b]	0.0100	0.0001	0.001	0.15
SUM			0.728	100

[a] Extraction with 95% ethanol, 80°C, 2 hours

[b] Extraction with 95% ethanol, 80°C, 3 hours

[c] Follow the spectrophotometric calibration curve (See Figure A-1 in Appendix A)

[d] Based on silk:solvent = 1:20
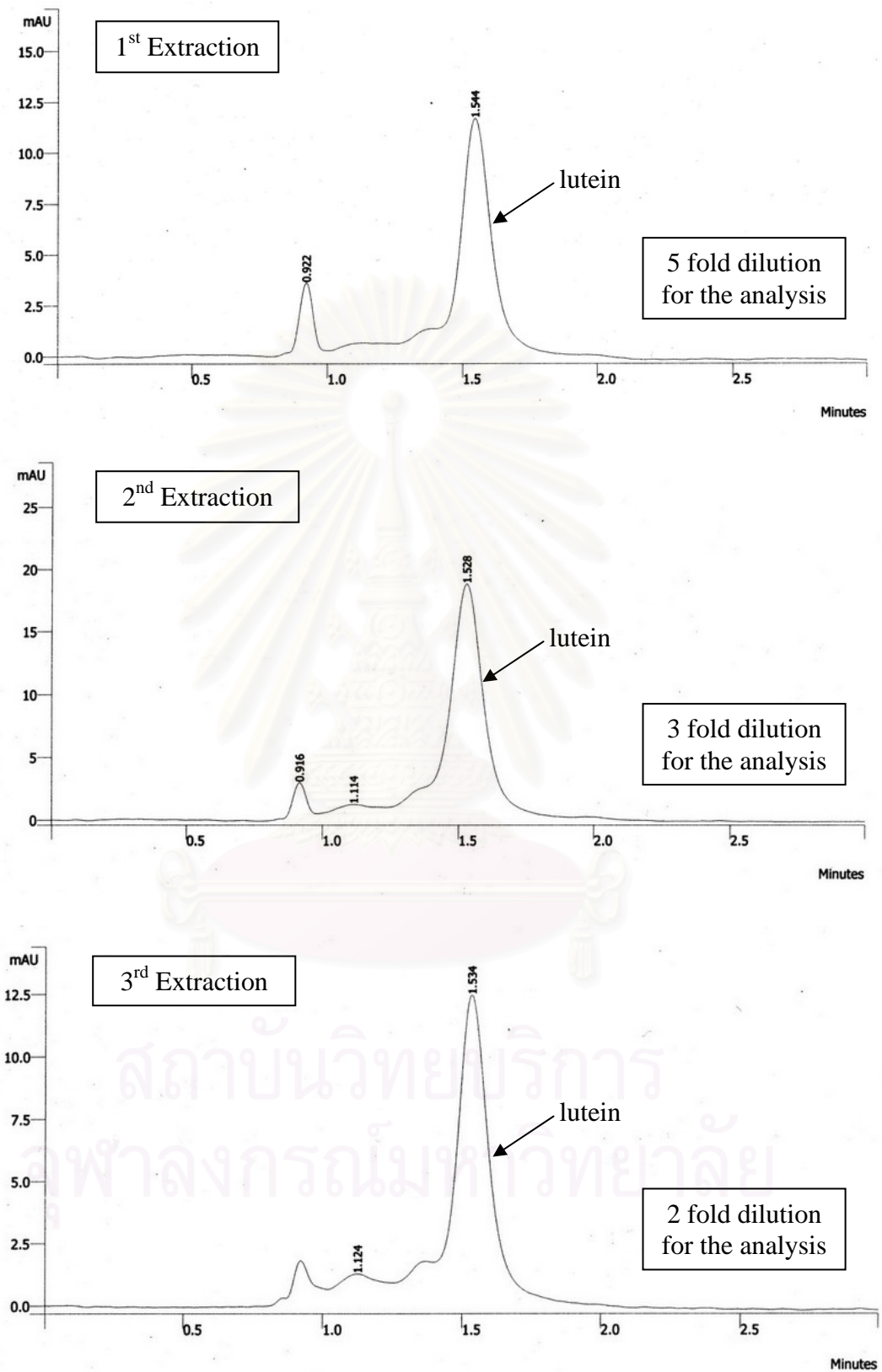
Figure 4.3 HPLC analysis of lutein in silk ethanolic extracts
(1st-3rd extraction of repeated batch system)
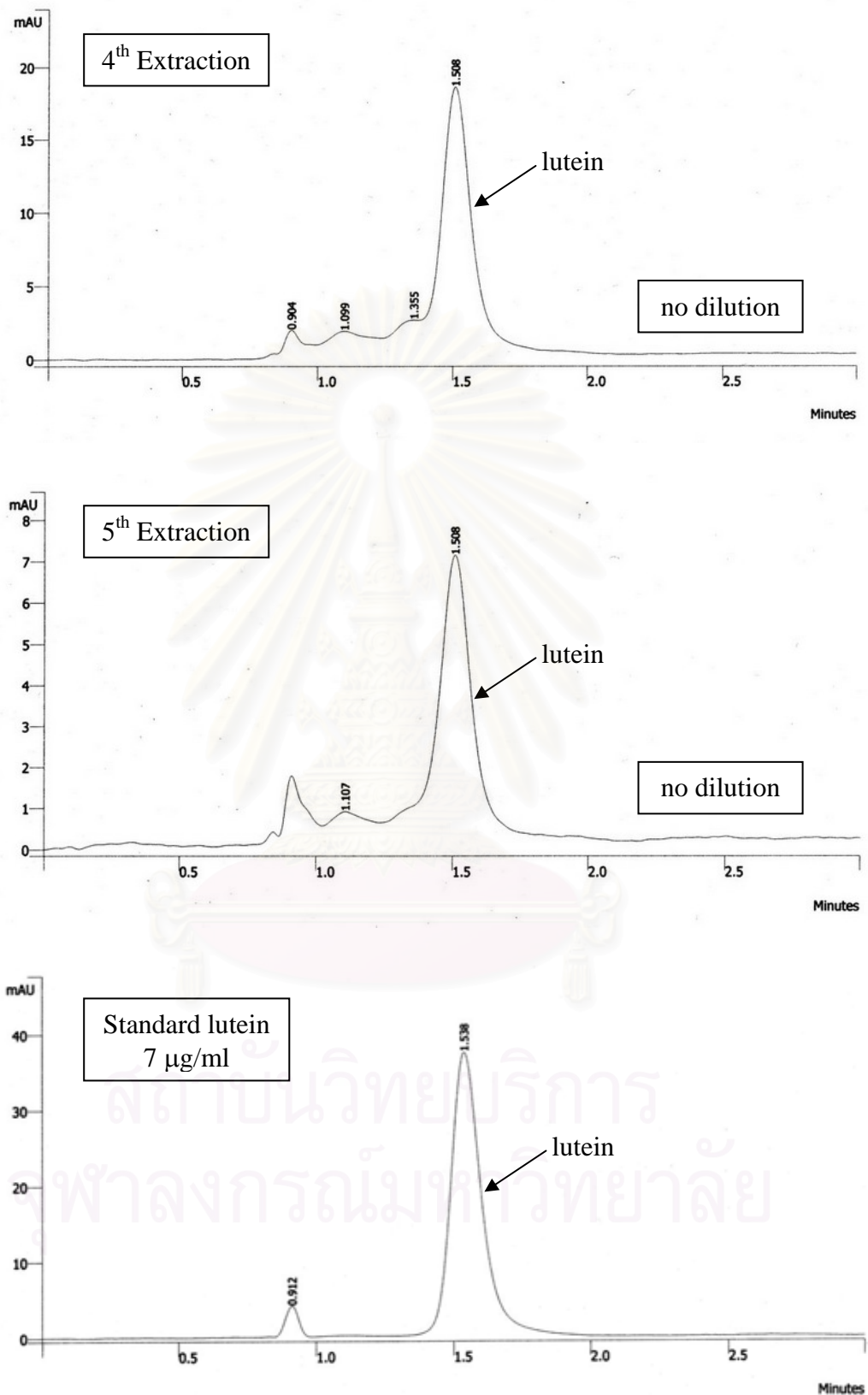
Figure 4.4 HPLC analysis of lutein in silk ethanolic extracts
(4th-5th extraction of repeated batch system) and standard lutein

For total flavonoids content, Table 4.2 shows that its content in the silk waste was 5.1 mg/g dry weight. This quantity is regarded as moderate content, compared to the amount of flavonoids in other fruits and vegetables such as onion skin (0.96 mg/g dry weight) [Wach et al., 2005], broccoli (3.04 mg/g dry weight), Italian kale (11.27 mg/g dry weight) [Heimler et al., 2006]. The HPLC analysis of the flavonoids is shown in figure 4.5, which the number of flavonoids and flavonoids derivatives are found. In this study, although only quercetin was identified against the standard compound, kaempferol and gucosides of kaempferol were reported to be other major components of flavonoids found in silk [Kurioka and Yamazaki, 2002].

Table 4.2 The content of total flavonoids in Thai yellow silk waste, Nangnoi race

No. of repeated batch	Abs 415 nm	Conc. of total flavonoids (mg/ml)[c]	Content of total flavonoids (mg/g dry weight)[d]	% of total flavonoids
1[a]	0.865	0.1276	2.552	50.04
2[a]	0.386	0.0569	1.138	22.30
3[b]	0.315	0.0464	0.928	18.19
4[b]	0.158	0.0233	0.466	9.14
5[b]	0.006	0.0008	0.016	0.32
SUM			5.100	100

[a] Extraction with 95% ethanol, $80^{o}C$, 2 hours

[b] Extraction with 95% ethanol, $80^{o}C$, 3 hours

[c] Followed the spectrophotometric calibration curve (See Figure A-2 in Appendix A)

[d] Based on silk:solvent = 1:20

It has been reported that, both carotenoids and flavonoids found in the silk waste are derived from the uptake of these compounds, present in the mulberry leaves, the diet of silk worm. For the carotenoids, the mechanism for the high-affinity selective uptake is believed to be caused by the carotenoid-binding proteins

[Tabunoki et al., 2002], and for *Bombyx mori*, the mechanism by which carotenoids are transported from the lumen of the midgut to the hemolymph, lipophorin, and from lipophorin into the silk gland, where the cocoon is produced, has been the primary focus [Jouni and Wells, 1996 and Tsuchida et al., 2004]. The mechanism of transport of flavonoids in silkworms has not yet been reported extensively. Nevertheless, some of the flavonoids glycosides found in silk were not found in the mulberry diet of the silk, which suggested that, unlike carotenoids, silkworms are able to synthesize flavonoids metabolites [Tamura et al., 2002].
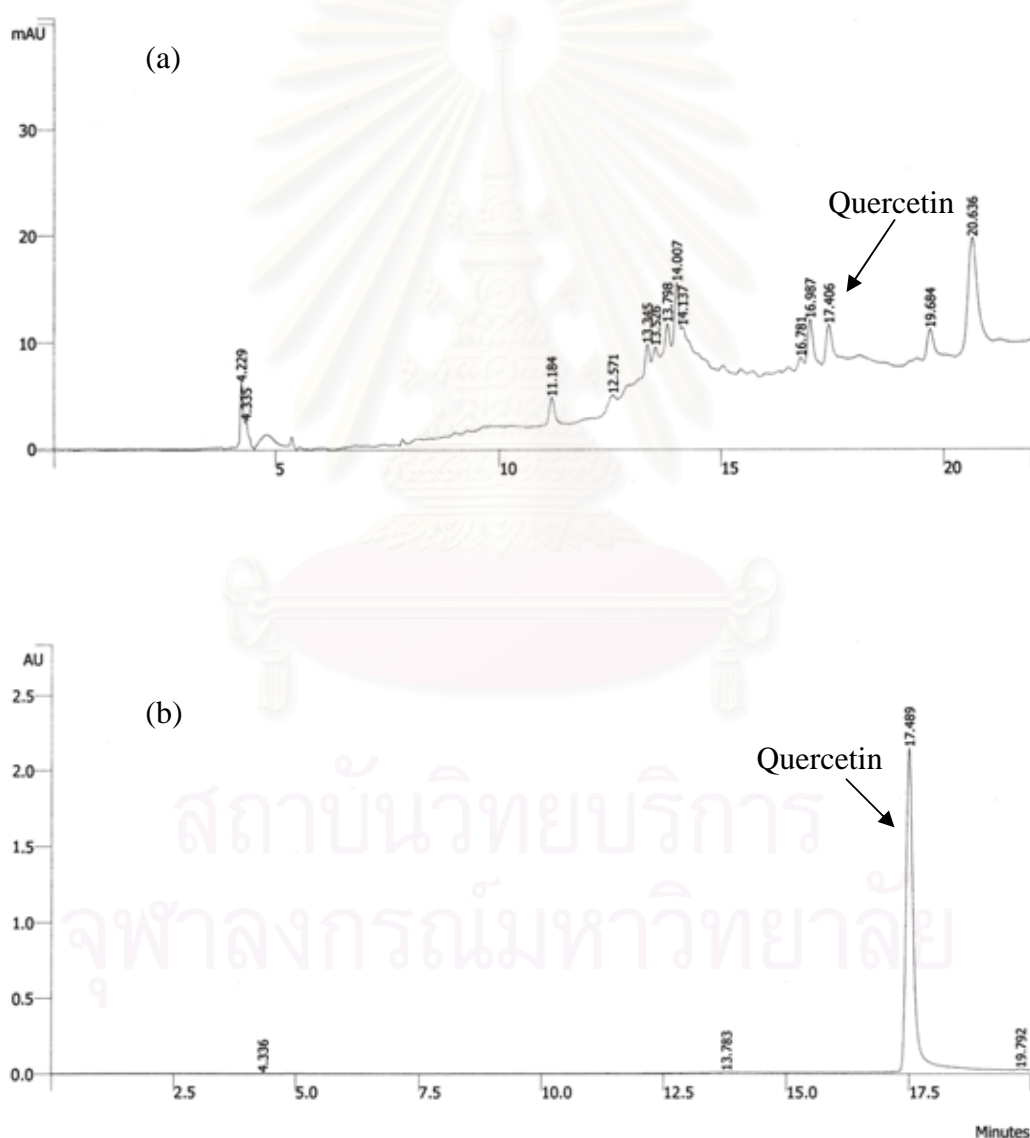
Figure 4.5 HPLC analysis of flavonoids in Thai yellow silk waste extract,

(a) ethanolic extract of Thai silk waste and (b) standard quercetin

4.2 Distribution of pigments in Thai yellow silk waste

In order to find the distribution of carotenoids and flavonoid on the sericin and fibroin, the layer of sericin was first removed by hydrolysis with subscritical water at 150^oC for 30 min in a pressure vessel. The remaining sample was that of fibroin was then extracted repeatedly with ethanol in the same system. The results are shown in Table 4.3. The layer of fibroin was found to contain approximately 0.44 mg of carotenoids/g dry weight and about 3.7 mg of flavonoids/g dry weight. The content of carotenoids in fibroin accounts for about 60% of the total pigment in silk waste sample (0.73 mg/g dry weight), while that of flavonoids located on fibroin accounts for more than 70% of the total pigment in silk waste sample (5.1 mg/g dry weight). It should be noted that the conditions employed for sericin removal might caused some pigments from fibroin to be extracted or could cause some degree of degradation of the compounds. Nevertheless, the conclusion about higher percentage of carotenoids and flavonoids contents in the fibroin could still be correctly drawn.

Table 4.3 Total carotenoids and flavonoids content on each layer, sericin and fibroin

	On sericin layer	On fibroin layer	SUM
Total carotenoids (mg/g dry weight)	0.287	0.441	0.728
Total flavanoids (mg/g dry weight)	1.435	3.670	5.105

4.3 Suitable solvents for extraction of the pigments.

Ethanol and subcritical water were investigated for benign extraction of carotenoids and flavonoids from silk waste. The results are presented as follows.

4.3.1 Extraction of silk carotenoids

The yellow silk waste was extracted with subcritical water at the temperatures of 120 and 150^oC and extraction times of 10 and 30 min. The results in Table 4.4

shows that the contents of total carotenoids extracted for all these conditions with subcritical water were not significantly different but was significantly lower than that obtained by ethanol extraction. Thus, ethanol was chosen as a suitable solvent for extraction of carotenoids and the profiles of ethanolic extraction of this pigment at various temperatures were studied. The results in Figure 4.7 shows that the content of carotenoids increased with increasing of temperature and reaction time.

Table 4.4 The contents of total carotenoids at selected conditions using subcritical water as a solvent.

Temperature (^{o}C)	Time (min)	Total carotenoids (mg/g dry weight)
120	10	0.0012
	30	0.0013
150	10	0.0016
	30	0.0015
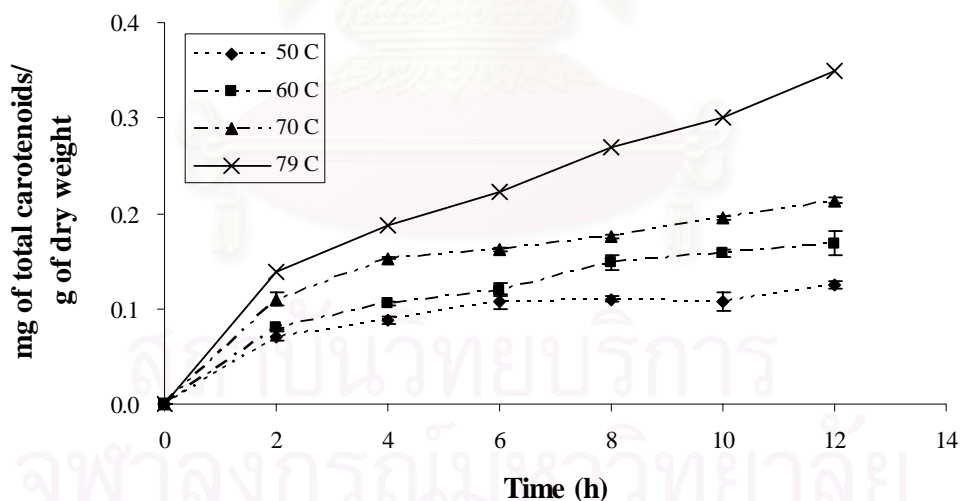
Figure 4.6 Effects of temperatures and reaction time on the contents of carotenoids

In addition, it was found that the contents of carotenoids were notably increased as the temperature was raised to 79°C, this is probably due to the fact that this temperature is the boiling point of the solvent, at which points the bubbles were formed and their random motion caused greater mixing for the system. For extraction

of carotenoids from silk which are mostly xanthophylls lutein, polar solvents, such as alcohols, acetone, or mixture of hexane and isopropyl alcohol are generally suggested as good extraction media due to the polar structure of the oxygenated groups at the ends of the carotenoid molecules [Delgado-Vargus et al., 2000]. Although the conditions of water could be adjusted so that its polarity is close to that of ethanol, it requires temperature as high as $200^{o}C$, which exceeds the degradation temperature of the carotenoids. Thus, the results in this suggested ethanol as a more suitable solvent for extraction of silk carotenoids.

4.3.2 Extraction of silk flavonoids

Figure 4.6 is the profiles of ethanolic extraction at various temperatures (50-79°C) and time of extraction. The contents of total flavonoids were found to increase with increasing of extraction time from 2-12 h and the contents of flavonoids extracted increased gradually with the rise of extraction temperature in the range of 50~70°C. This is probably due to the increased in solubility and the fact that the greater speed of the molecular movements at higher temperature caused the extracting agent to diffuse more quickly into the silk sample to extract the flavonoids, and caused the flavonoids to diffuse more quickly from the silk sample to extracting agent. It could be seen from Figure 4.6 that at the temperature surpassing 70°C, the
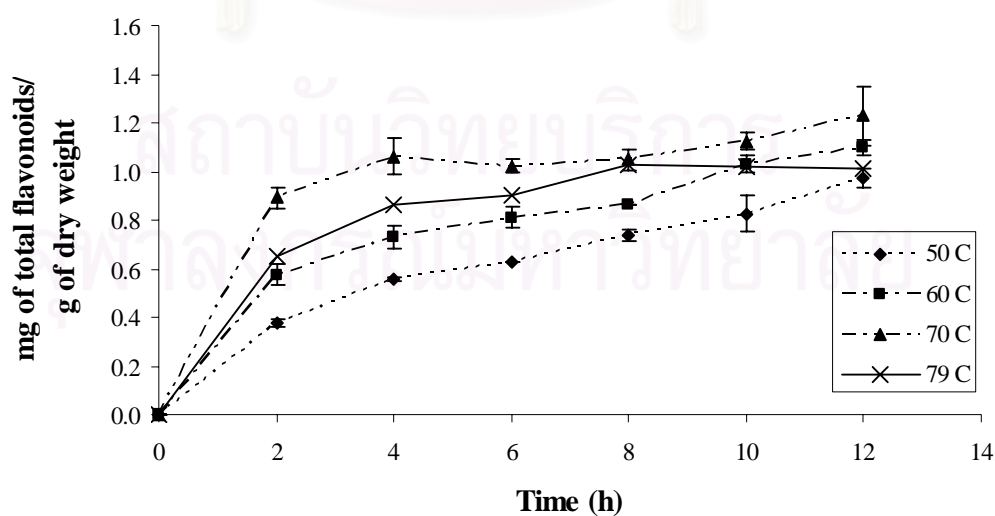
Figure 4.7 Effect of ethanolic extraction time and temperature

on the contents of extracted flavonoids

total flavonoids extracted started to decrease due to the compound degradation at high temperature.

Besides, ethanolic extraction, subcritical water extractions of Thai yellow silk waste at the temperature of 120-150°C with the reaction time of 10-60 min were carried out to study the effect of subcritical water conditions on the content of flavonoids and the result was shown in Figure 4.7. Flavonoids content extracted from subcritical water increased with   increasing extraction time and temperature. However, subcritical water extraction at temperature in the range of 120-140°C, higher amount of flavonoids was extracted with shorter extraction time (10 min). Moreover, the flavonoids content in the water extracts was higher than that in the ethanolic extracts even at longest extraction time (12 h), which indicated that water is more suitable extraction solvent than ethanol for flavonoids. High solubility of
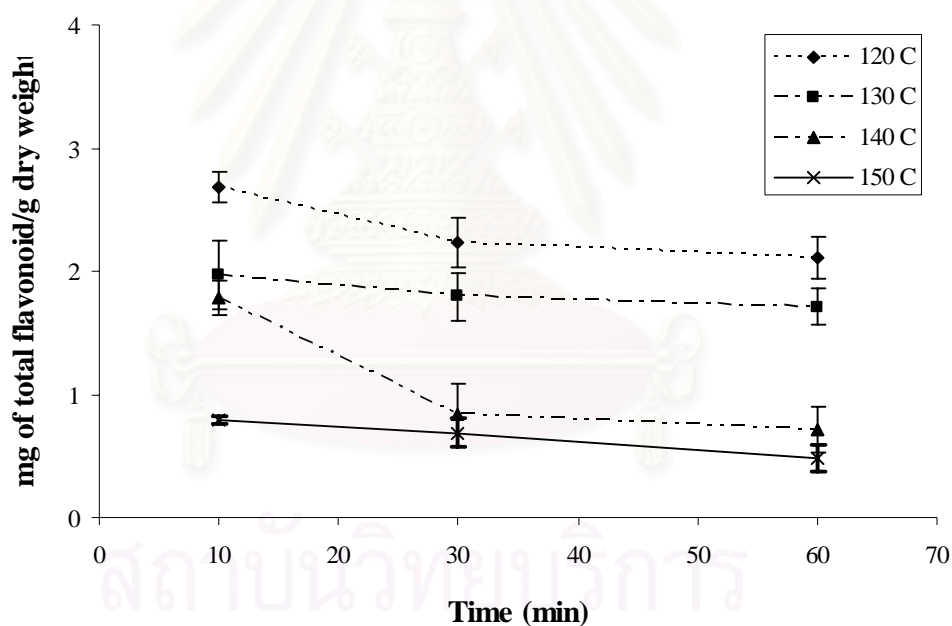
Figure 4.8 Effect of ethanolic extraction time and temperature
on the contents of extracted flavonoids

flavonoids in water is possibly due to the fact that the compounds contains many hydroxyl groups, and that also that flavonoids are mostly present mainly as glycosides whose molecules are attached with different mono-, di-, or trisaccharides or combinations of these, which can bind to the different hydroxyl groups of the flavonoids aglycone [Tamura et al., 2001 and Kurioka and Yamazaki, 2002].

4.4 Antioxidant activity of the extracts

In previous studies, silk sericin has been reported to exhibit antioxidant activity and has been used for various pharmacuetical and cosmecuetical applications. However, due to the fact sericin protein was not the only component of the subcritical water extract, and that this solution contain some amount of yellow pigments, in this study, the antioxidant activity of subcritical water extract of Thai yellow silk waste, the subcritical water extract of silk waste whose pigments had previously been extracted, and the ethanol extract of silk waste were determined.

The ABTS antioxidant activity assay was used and the activities of various extracts were compared based on the value of IC_{50}, the concentration of sample producing 50% reduction of the radical absorbance. Figure 4.8 shows that IC_{50} of the extracts whose yellow pigment was first removed, was higher than that of the value of the water extracts obtained from the yellow silk waste. This means that the antioxidant activity of the sericin extract (without pigment) was lower than that of the extract contained a small amount of pigments. This could be implied that the pigments possibly be higher effective antioxidant compound than protein sericin. Since the pigments in the extracts of raw yellow silk constitutes only about 3 wt% but the the antioxidant activities was twice increased when this small amount of pigment was removed, this implied then that the antioxidant activity of the pigment was much higher than that of the sericin alone. This was then verified by the examination of antioxidant activity of ethanolic extracts of yellow silk, which contained a minimal amount of sericin. Figure 4.9 shows that the IC_{50} of the ethanolic extracts obtained at various temperatures (50-79°C) lie in the range of 15.6-23.3 μg/ml which indicated that this extracts are high antioxidant compound compared to IC_{50} of some medicinal plants and tropical fruits in table 2.7 [Silva et al., 2005] and 2.8 [Lim et al., 2006], respectively. In addition, from this result, in which the IC_{50} of ethanolic extracts was significantly lower than that of subcritical water extracts, it could be confirmed that the pigments in yellow silk play an important role in exhibiting the antioxidant activity.
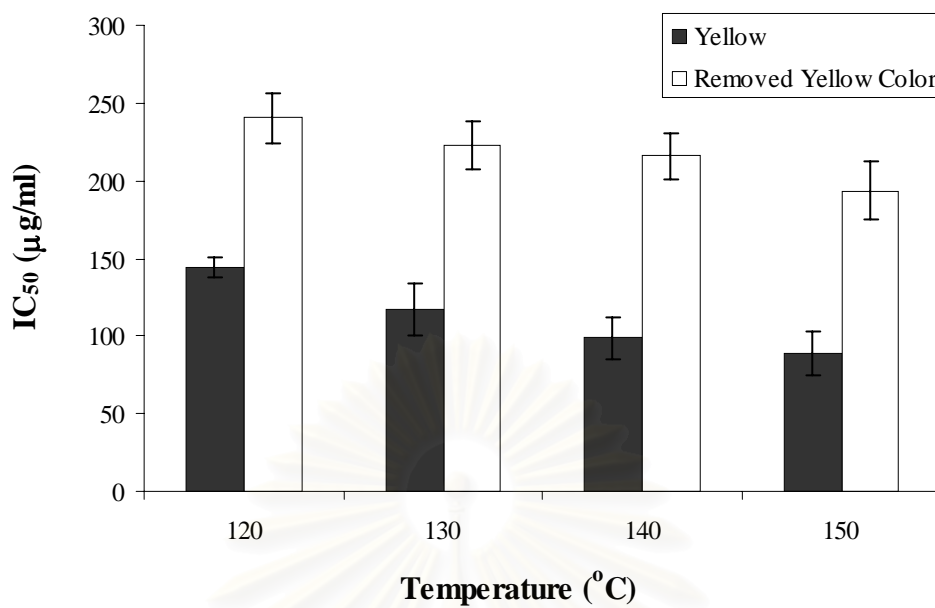
Figure 4.9 Antioxidant activity of subcritical water extracts

T (^{o}C)	IC$_{50}$ (µg/ml)
50	15.608
60	19.022
70	19.436
80	23.334
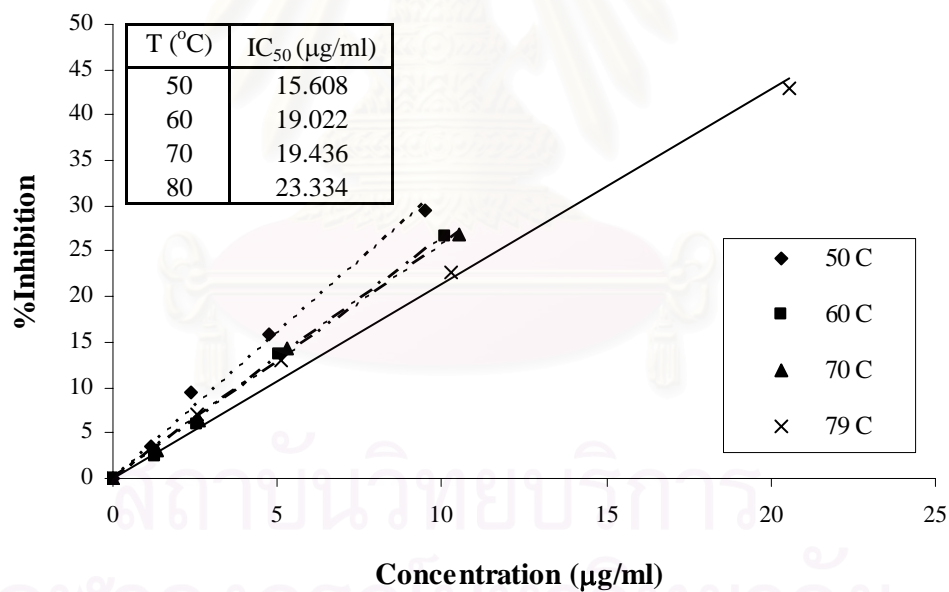
Figure 4.10 Antioxidant activity of ethanolic extracts

CHAPTER V

CONCLUSIONS AND RECOMMENDATIONS

5.1 Conclusions

5.1.1 This study demonstrated that a yellow pigment covering Thai silk waste, Nangnoi could be completely removed with five times of 2-3 hours repeated batch at 80°C using ethanol as solvent. The total carotenoids and flavonoids presented in the extracts were 0.7 and 5.1 mg/g dry silk, respectively.

5.1.2 About 60% of carotenoids and more than 70% of flavonoids locate on fibroin layer of the silk waste.

5.1.3 The suitable environmentally benign solvent for the recovery of carotenoids is ethanol and that of flavonoids is water.

5.1.4 Temperature and extraction time are the two parameters affecting the yield of total carotenoids in ethanolic extracts as the yield increased with the increasing of temperature (from 50 to 79°C) and extraction time (from 2-12 hours). Temperature and time also affected the yield of flavonoids, which increased with increasing temperature from 50 to 70°C and then decreased above 79°C due to thermal degradation.

5.1.5 The extracts of yellow pigment of Thai silk waste, Nangnoi race, show effective antioxidant activity. The concentration of the extract that reduces the redical absorbance to 50% was 15.5-23.3 μg/ml.

5.2 Recommendations

5.2.1 For future studies, more detailed analysis of the products should be conducted using high performance liquid chromatography (HPLC) in order to determine the amount of flavonoids and carotenoids particularly lutein which was notably found in the extract.

5.2.2 In the present work, the yield of carotenoids increased with increasing temperature from 50 to 79°C which is the boiling point of ethanol at

atmospheric pressure. Since we was informed from this study that the degradation temperature of carotenoids was higher than 140°C, therefore, the ethanolic extraction of carotenoids from yellow silk is interested to carried on higher temperature (above boiling point, 79°C) operating with the pressurized system. Conformational changes of the structures as a results of temperature should be observed.

5.2.3 Other organic solvent and their mixtures should be investigated in order to optimize the extraction process.

5.2.4 For the applications, due to these products, carotenoids and flavonoids are associated with the medicinal functions, therefore more information about their structures, activities, including the toxicity should essentially be studied.

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APPENDICES

APPENDIX A

EXPERIMENTAL AND DATA ANALYSIS

A-1 Standard calibration curve of total carotenoids

Table A-1 Standard calibration data of total carotenoids

Concentration of lutein (mg/ml)	Absorbance at 478 nm
0.0	0.000
1.0	0.186
2.0	0.336
3.0	0.534
4.0	0.622
4.5	0.839
5.0	0.932
6.0	1.061
7.0	1.262
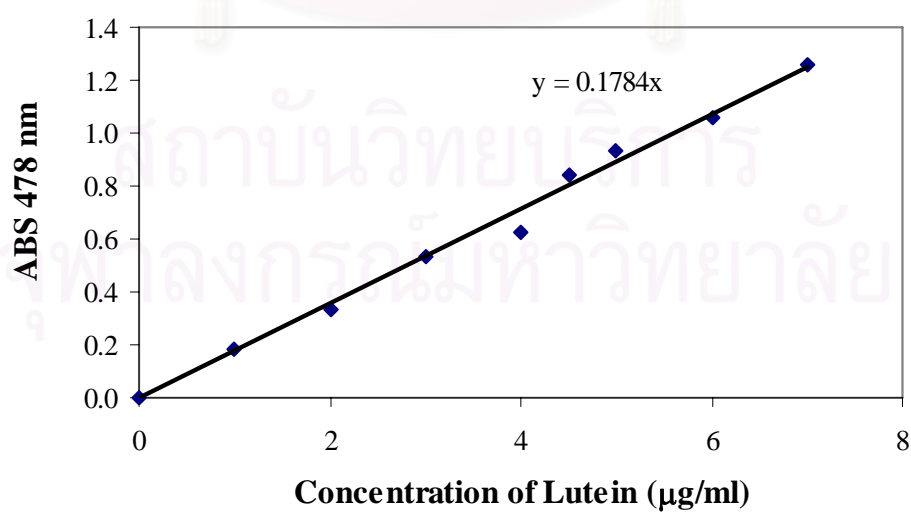
Figure A-1 Standard calibration curve of lutein

A-2 Standard calibration curve of total flavonoids

Table A-2 Standard calibration data of total flavonoids

Concentration of quercetin (µg/ml)	Absorbance at 415 nm.			
	Exp.1	Exp.2	Exp.3	Average
0.0	0.000	0.000	0.000	0.000
12.5	0.094	0.092	0.095	0.094
25.0	0.158	0.165	0.169	0.164
50.0	0.294	0.298	0.315	0.302
80.0	0.580	0.580	0.603	0.580
100.0	0.558	0.658	0.665	0.662
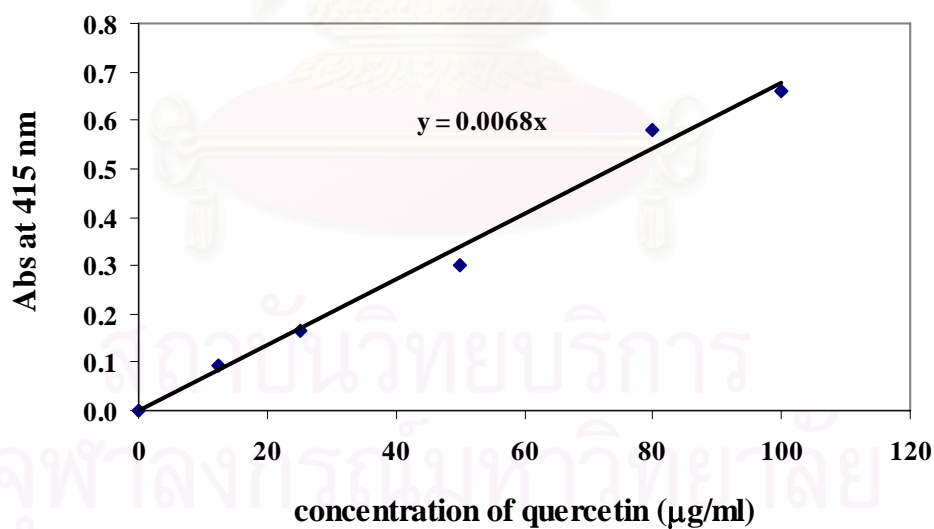
Figure A-2 Standard calibration curve of quercetin

A-3 HPLC analysis of standard lutein

Table A-3 Standard calibration data of lutein

Concenteation of lutein (μg/ml)	Peak Area
0.0	0
1.0	332525
2.0	720018
3.0	1010839
4.0	1489528
4.5	2081387
5.0	2316044
6.0	2746215
7.0	2956062
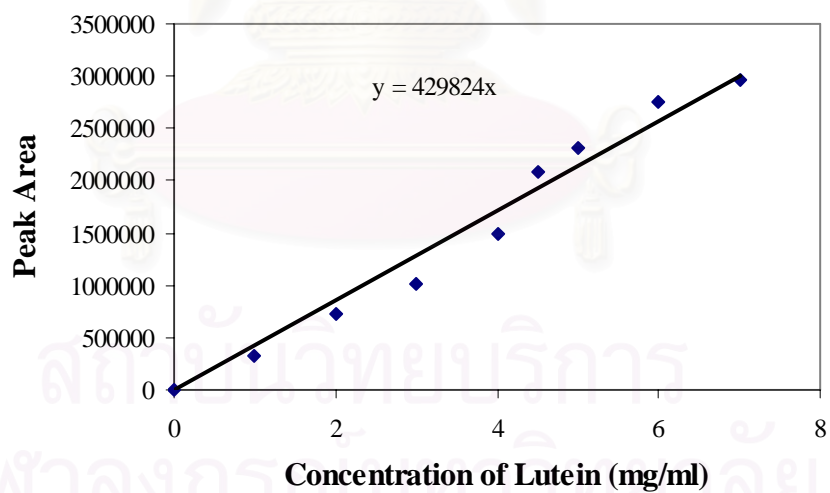
Figure A-3 Standard calibration curve for HPLC analysis of lutein

APPENDIX B

EXPERIMENTAL DATA

B-1 The data of recovery of the pigments on yellow silk waste

Table B-1.1 Total carotenoids content on repeated batch ethanolic extracts

No.of repeated batch	Temperature/Time	Total carotenoids (mg/ml)			
		Exp.1	Exp.2	Average	SD
1	80°C, 2 h	0.324	0.629	0.476	0.215
2	80°C, 2 h	0.192	0.180	0.186	0.009
3	80°C, 3 h	0.059	0.031	0.045	0.020
4	80°C, 3 h	0.029	0.010	0.019	0.013
5	80°C, 3 h	0.001	0.001	0.001	0.000
SUM		0.605	0.851	0.728	

Table B-1.2 Total flavonoids content on repeated batch ethanolic extracts

No.of repeated batch	Temperature/Time	Total flavonoids (mg/g db.)			
		Exp.1	Exp.2	Average	SD
1	80°C, 2 h	0.324	0.62	2.555	0.375
2	80°C, 2 h	0.192	1.25	1.140	0.156
3	80°C, 3 h	0.059	0.91	0.930	0.028
4	80°C, 3 h	0.029	0.47	0.465	0.007
5	80°C, 3 h	0.001	0.02	0.015	0.007
SUM		0.605	5.47	5.105	

B-2 The data of pigments on fibroin layer.

Table B-2.1 Total carotenoids content on repeated batch ethanolic extracts

No.of repeated batch	Temperature/Time	Total carotenoids (mg/ml)			
		Exp.1	Exp.2	Average	SD
1	80°C, 2 h	0.357	0.339	0.348	0.012
2	80°C, 2 h	0.063	0.039	0.051	0.017
3	80°C, 3 h	0.026	0.005	0.016	0.014
4	80°C, 3 h	0.005	0.003	0.004	0.002
5	80°C, 3 h	0.002	0.043	0.023	0.029
SUM		0.453	0.429	0.441	

Table B-2.2 Total flavonoids content on repeated batch ethanolic extracts

No.of repeated batch	Temperature/Time	Total flavonoids (mg/g db.)			
		Exp.1	Exp.2	Average	SD
1	80°C, 2 h	2.89	3.01	2.950	0.085
2	80°C, 2 h	0.48	0.42	0.450	0.042
3	80°C, 3 h	0.12	0.21	0.165	0.064
4	80°C, 3 h	0.04	0.08	0.060	0.028
5	80°C, 3 h	0.03	0.06	0.045	0.021
SUM		3.56	3.78	3.670	

B-3 Experimental data of ethanolic extraction

Table B-3.1 Content of total carotenoids on ethanolic extraction at 50°C and the reaction time of 2-12 h

Temperature (^{o}C)	Time (h)	Total carotenoids (mg/g dry weight.)			
		Exp.1	Exp.2	Average	SD
50	0	0.000	0.000	0.000	0.000
	2	0.067	0.074	0.070	0.005
	4	0.086	0.091	0.088	0.004
	6	0.102	0.112	0.107	0.007
	8	0.107	0.112	0.110	0.004
	10	0.101	0.114	0.107	0.009
	12	0.128	0.122	0.125	0.004

Table B-3.2 Content of total carotenoids on ethanolic extraction at 60°C and the reaction time of 2-12 h

Temperature (^{o}C)	Time (h)	Total carotenoids (mg/g dry weight.)			
		Exp.1	Exp.2	Average	SD
60	0	0.000	0.000	0.000	0.000
	2	0.079	0.081	0.080	0.001
	4	0.107	0.104	0.106	0.002
	6	0.124	0.116	0.120	0.006
	8	0.154	0.143	0.148	0.008
	10	0.161	0.156	0.159	0.004
	12	0.178	0.159	0.169	0.013

Table B-3.3 Content of total carotenoids on ethanolic extraction at 70°C and the reaction time of 2-12 h

Temperature (^{o}C)	Time (h)	Total carotenoids (mg/g dry weight.)			
		Exp.1	Exp.2	Average	SD
70	0	0.000	0.000	0.000	0.000
	2	0.105	0.115	0.110	0.007
	4	0.152	0.154	0.153	0.001
	6	0.161	0.164	0.162	0.002
	8	0.178	0.175	0.176	0.002
	10	0.196	0.193	0.194	0.002
	12	0.211	0.215	0.213	0.003

Table B-3.4 Content of total carotenoids on ethanolic extraction at 79°C and the reaction time of 2-12 h

Temperature (^{o}C)	Time (h)	Total carotenoids (mg/g dry weight.)			
		Exp.1	Exp.2	Average	SD
79	0	0.000	0.000	0.000	0.000
	2	0.138	0.138	0.138	0.000
	4	0.188	0.188	0.188	0.000
	6	0.223	0.223	0.223	0.000
	8	0.268	0.268	0.268	0.000
	10	0.300	0.300	0.300	0.000
	12	0.349	0.349	0.349	0.000

Table B-3.5 Content of flavonoids on ethanolic extraction at 50°C and the reaction time of 2-12 h

Temperature ($^{\circ}$C)	Time (h)	Total flavonoids (mg/g db.)			
		Exp.1	Exp.2	Average	SD
	0	0.00	0.00	0.000	0.000
	2	0.39	0.64	0.515	0.175
	4	0.56	0.55	0.557	0.006
50	6	0.63	0.63	0.627	0.000
	8	0.75	0.72	0.738	0.021
	10	0.88	0.78	0.827	0.073
	12	0.94	1.00	0.974	0.042

Table B-3.5 Content of flavonoids on ethanolic extraction at 60°C and the reaction time of 2-12 h

Temperature ($^{\circ}$C)	Time (h)	Total flavonoids (mg/g db.)			
		Exp.1	Exp.2	Average	SD
	0	0.00	0.00	0.000	0.000
	2	0.61	0.55	0.576	0.042
	4	0.69	0.76	0.727	0.047
60	6	0.84	0.78	0.812	0.042
	8	0.86	0.86	0.863	0.000
	10	1.00	1.06	1.029	0.037
	12	1.08	1.12	1.099	0.031

Table B-3.5 Content of flavonoids on ethanolic extraction at 70°C and the reaction time of 2-12 h

Temperature (°C)	Time (h)	Total flavonoids (mg/g db.)			
		Exp.1	Exp.2	Average	SD
70	0	0.00	0.00	0.000	0.000
	2	0.86	0.92	0.893	0.042
	4	1.14	1.01	1.076	0.091
	6	1.04	1.00	1.022	0.026
	8	1.02	1.08	1.048	0.042
	10	1.10	1.15	1.125	0.037
	12	1.14	1.31	1.228	0.120

Table B-3.6 Content of flavonoids on ethanolic extraction at 79°C and the reaction time of 2-12 h

Temperature (°C)	Time (h)	Total flavonoids (mg/g db.)			
		Exp.1	Exp.2	Average	SD
79	0	0.00	0.00	0.000	0.000
	2	0.65	0.65	0.649	0.000
	4	0.86	0.86	0.863	0.000
	6	0.90	0.90	0.900	0.000
	8	1.03	1.03	1.025	0.000
	10	1.02	1.02	1.018	0.000
	12	1.01	1.01	1.011	0.000

B-4 Experimental data of subcritical water extraction

Table B-4.1 Content of total flavonoids on subcritical water extraction at 120-150°C and the reaction time of 10, 30 and 60 min

Temperature (^{o}C)	Time (min)	Total Flavonoids (mg/g db.)			
		Exp.1	Exp.2	Average	SD
120	10	2.60	2.76	2.682	0.117
	30	2.38	2.10	2.237	0.200
	60	2.23	1.99	2.114	0.169
130	10	2.16	1.77	1.968	0.275
	30	1.93	1.65	1.791	0.200
	60	1.82	1.61	1.713	0.148
140	10	1.89	1.69	1.787	0.140
	30	1.02	0.65	0.834	0.257
	60	0.84	0.58	0.707	0.186
150	10	0.77	0.81	0.791	0.025
	30	0.60	0.77	0.688	0.117
	60	0.40	0.55	0.477	0.111

B-5 Experimental data of antioxidant activity

Table B-5.1 Percent inhibition of 10 min SWE of yellow silk waste at various temperature, 120-150°C

Time (min)	T (^{o}C)		Exp.1						Exp.2					
10	120	µg/ml	0.00	39.99	79.97	159.95	319.89	639.78	0.00	38.98	77.96	155.91	311.83	623.66
		% Inhibition	0.00	19.37	33.78	52.85	73.27	92.64	0.00	23.29	33.33	46.43	67.39	83.99
	130	µg/ml	0.00	46.03	92.06	184.11	368.23	736.46	0.00	41.33	82.66	165.32	330.65	661.29
		% Inhibition	0.00	23.72	42.64	61.41	83.33	97.30	0.00	27.07	44.10	55.31	71.62	85.01
	140	µg/ml	0.00	48.54	97.07	194.15	388.29	776.58	0.00	43.35	86.69	173.39	346.77	693.55
		% Inhibition	0.00	36.23	47.90	77.10	90.87	97.90	0.00	34.93	51.67	70.45	82.53	89.96
	150	µg/ml	0.00	50.02	100.03	200.06	400.12	800.25	0.00	36.29	72.58	145.16	290.32	580.65
		% Inhibition	0.00	37.43	58.38	69.31	92.96	98.35	0.00	26.96	45.63	67.17	89.76	94.43
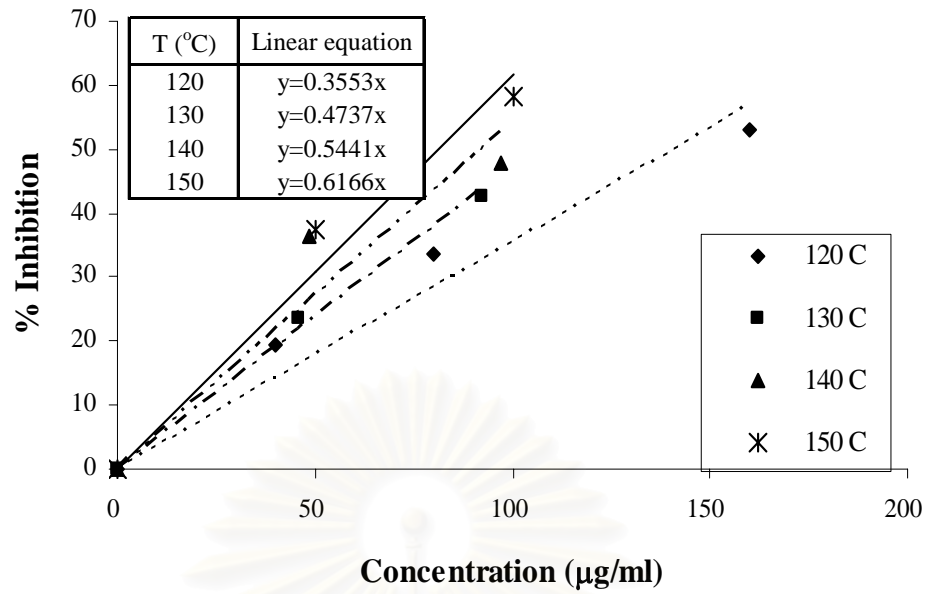
(a)
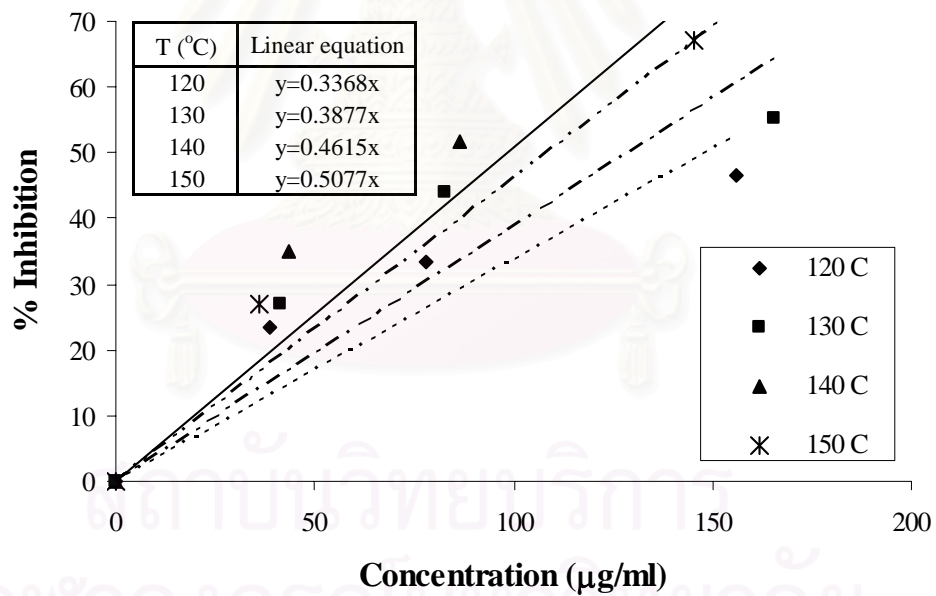
(b)

Figure B-5.1 Percent inhibition of 10 min SWE of yellow silk waste at various temperature, 120-150°C, (a) Exp.1 and (b) Exp.2
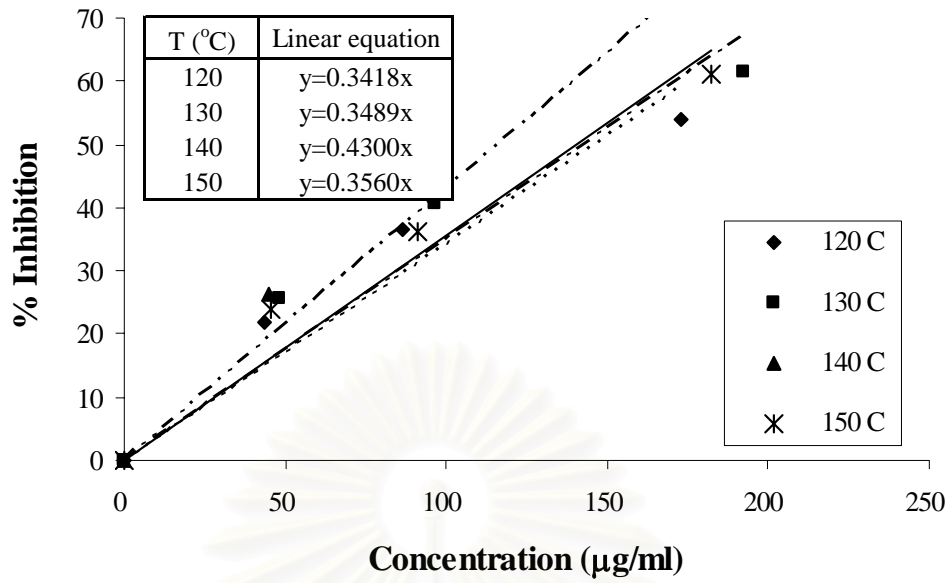
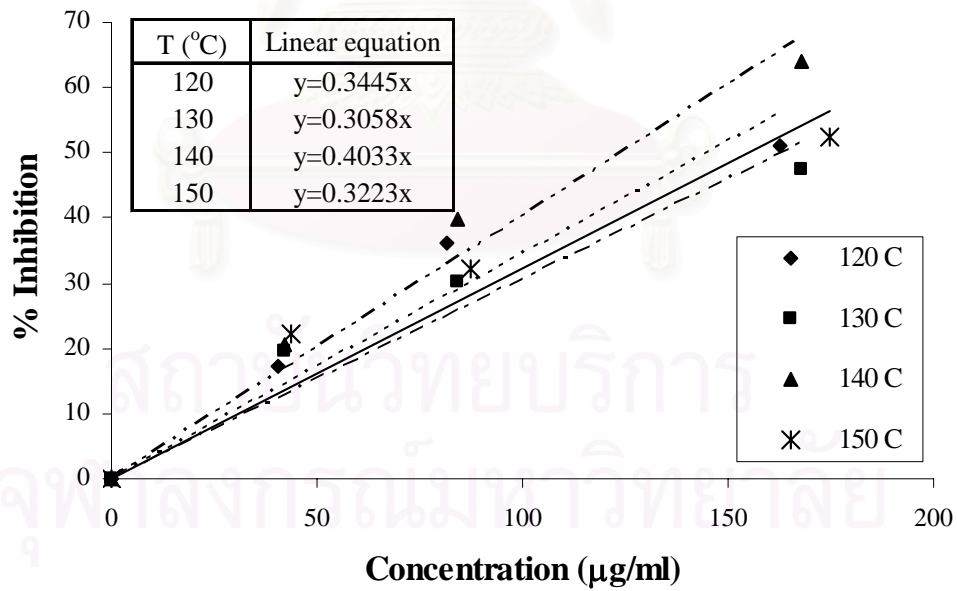**Table B-5.2 Percent inhibition of 30 min SWE of yellow silk waste at various temperature, 120-150°C**

Time (min)	T (^{o}C)		Exp.1						Exp.2					
30	120	µg/ml	0.00	43.26	86.51	173.02	346.04	692.08	0.00	40.66	81.32	162.63	325.27	650.54
		% Inhibition	0.00	21.92	36.49	53.90	68.62	82.88	0.00	14.71	34.23	49.55	71.62	84.68
	130	µg/ml	0.00	48.16	96.33	192.65	385.30	770.61	0.00	42.00	84.01	168.01	336.02	672.04
		% Inhibition	0.00	25.53	40.54	61.56	82.13	96.85	0.00	17.12	27.93	45.80	68.47	82.88
	140	µg/ml	0.00	44.89	89.77	179.55	359.10	718.20	0.00	42.00	84.01	168.01	336.02	672.04
		% Inhibition	0.00	26.33	46.42	71.54	84.02	91.93	0.00	17.05	36.99	62.25	70.78	89.35
	150	µg/ml	0.00	45.56	91.13	182.26	364.52	729.03	0.00	43.68	87.37	174.73	349.46	698.92
		% Inhibition	0.00	23.74	36.07	61.19	68.34	83.11	0.00	21.31	31.51	51.75	73.82	91.93

(a)

(b)

Figure B-5.2 Percent inhibition of 30 min SWE of yellow silk waste at various

temperature, 120-150°C, (a) Exp.1 and (b) Exp.2

Table B-5.3 Percent inhibition of 60 min SWE of yellow silk waste at various temperature, 120-150°C

Time (min)	T (^{o}C)		Exp.1						Exp.2					
60	120	µg/ml	0.00	45.11	90.21	180.43	360.85	721.71	0.00	42.34	84.68	169.35	338.71	677.42
		% Inhibition	0.00	37.57	52.40	69.76	88.17	97.75	0.00	33.23	50.15	68.41	84.28	89.67
	130	µg/ml	0.00	52.97	105.95	211.89	423.78	847.56	0.00	42.34	84.68	169.35	338.71	677.42
		% Inhibition	0.00	37.72	55.54	71.71	91.32	97.31	0.00	33.08	45.21	62.57	73.65	84.58
	140	µg/ml	0.00	45.16	90.32	180.65	361.29	722.58	0.00	41.67	83.33	166.67	333.33	666.67
		% Inhibition	0.00	20.24	31.51	54.64	72.45	86.91	0.00	17.05	27.55	43.07	69.71	91.02
	150	µg/ml	0.00	45.75	91.50	183.00	366.00	732.01	0.00	45.03	90.05	180.11	360.22	720.43
		% Inhibition	0.00	22.22	35.77	51.90	69.56	83.87	0.00	27.40	39.42	54.49	75.80	95.43

(a)

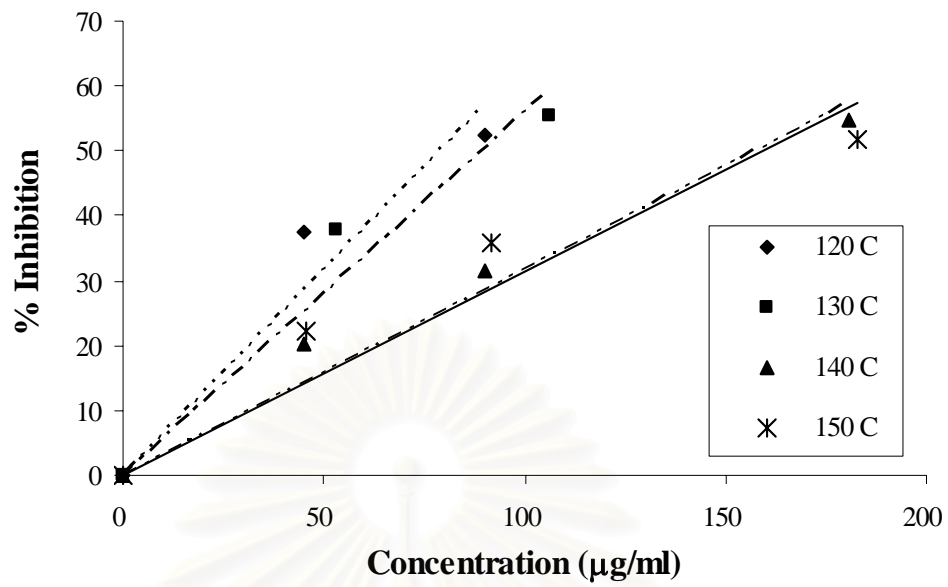| T (^{o}C)	Linear equation
120	y=0.6525x
130	y=0.6065x
140	y=0.2849x
150	y=0.3471x

(b)

Figure B-5.3 Percent inhibition of 60 min SWE of yellow silk waste at various
temperature, 120-150°C, (a) Exp.1 and (b) Exp.2

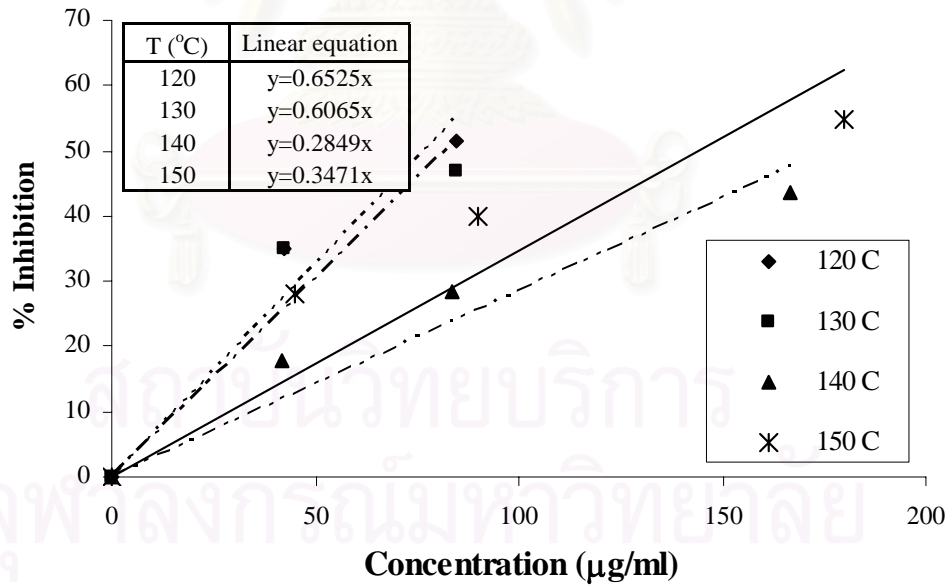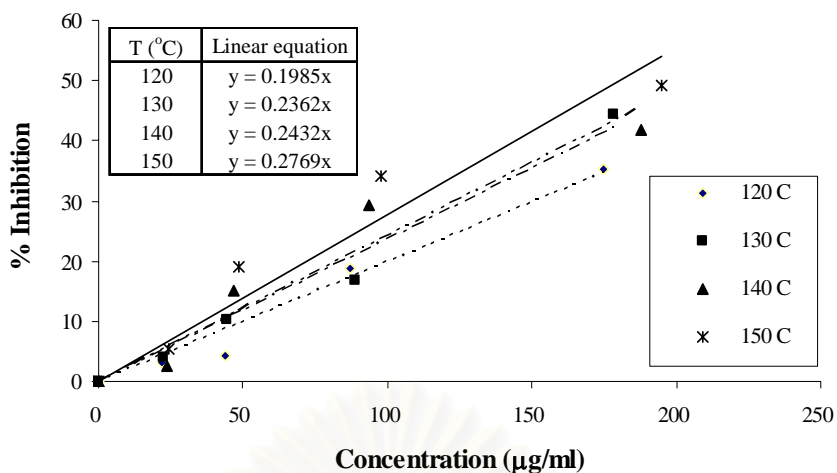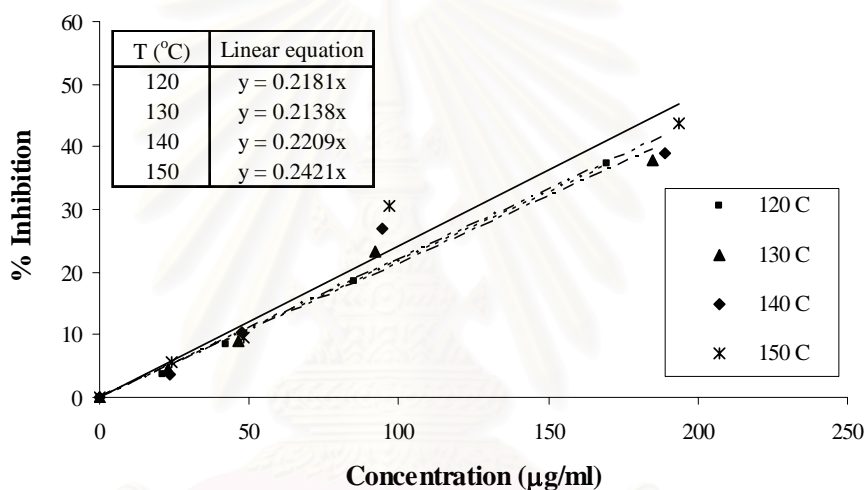**Table B-5.4 IC_{50} of SWE of yellow silk waste at various temperature, 120-150°C**

Time (min)	T (^{o}C)	Linear equation		IC_{50} (mg/ml)			
		Exp.1	Exp.2	Exp.1	Exp.2	Average	SD
10	120	y=0.3553x	y=0.3368x	140.73	148.46	144.59	5.47
	130	y=0.4737x	y=0.3877x	105.55	128.97	117.26	16.56
	140	y=0.5441x	y=0.4615x	92.42	108.34	100.38	11.26
	150	y=0.6166x	y=0.5077x	81.09	98.48	89.79	12.30
30	120	y=0.3418x	y=0.3445x	146.28	145.14	145.71	0.81
	130	y=0.3489x	y=0.3058x	143.31	163.51	153.41	14.28
	140	y=0.4300x	y=0.4033x	116.28	123.98	120.13	5.44
	150	y=0.3560x	y=0.3223x	140.45	155.13	147.79	10.38
60	120	y=0.6312x	y=0.6525x	79.21	76.63	77.92	1.83
	130	y=0.5618x	y=0.6065x	89.00	82.44	85.72	4.64
	140	y=0.3183x	y=0.2849x	157.08	175.50	166.29	13.02
	150	y=0.3137x	y=0.3471x	159.39	144.05	151.72	10.85

Table B-5.5 Percent inhibition of 10 min SWE of yellow removed silk waste at various temperature, 120-150°C

Exp.	T (^{o}C)		Experimental data						
1	120	µg/ml	0.00	21.84	43.68	87.37	174.73	349.46	698.92
		% Inhibition	0.00	3.22	4.18	18.81	35.21	82.64	91.16
	130	µg/ml	0.00	22.26	44.52	89.05	178.09	356.18	712.37
		% Inhibition	0.00	3.86	10.29	16.88	44.37	87.94	93.89
	140	µg/ml	0.00	23.46	46.92	93.84	187.68	375.37	750.73
		% Inhibition	0.00	2.49	14.95	29.40	41.86	83.39	94.85
	150	µg/ml	0.00	24.38	48.75	97.51	195.01	390.03	780.06
		% Inhibition	0.00	5.32	19.10	34.22	49.17	88.54	95.02
2	120	µg/ml	0.00	21.21	42.42	84.85	169.69	339.38	678.76
		% Inhibition	0.00	3.51	8.54	18.45	37.35	73.48	92.38
	130	µg/ml	0.00	23.10	46.20	92.41	184.81	369.62	739.25
		% Inhibition	0.00	4.55	9.10	23.37	37.94	69.65	94.39
	140	µg/ml	0.00	23.64	47.29	94.57	189.15	378.30	756.60
		% Inhibition	0.00	3.64	10.47	26.86	39.00	57.81	96.97
	150	µg/ml	0.00	24.19	48.39	96.77	193.55	387.10	774.19
		% Inhibition	0.00	5.52	9.66	30.52	43.87	57.36	96.47

(a)

(b)

Figure B-5.4 IC$_{50}$ of SWE of 10 min removed yellow silk waste at various temperature, 120-150°C, (a) Exp.1 and (b) Exp.2

Table B-5.6 IC$_{50}$ of SWE of 10 min removed yellow silk waste at various temperature, 120-150°C

Time (min)	T (^{o}C)	Linear equation		IC$_{50}$ (μg/ml)			
		Exp.1	Exp.2	Exp.1	Exp.2	Average	SD
10	120	y = 6.40x	y = 7.03x	7.81	7.11	7.46	0.50
	130	y = 7.62x	y = 6.90x	6.56	7.25	6.91	0.49
	140	y = 7.84x	y = 7.13	6.37	7.02	6.70	0.45
	150	y = 8.93x	y = 7.80x	5.60	6.40	6.00	0.57

Table B-5.7 Percent inhibition of 4 hours ethanolic extraction of yellow silk waste at various temperature, 50-79°C

Time (h)	T (^{o}C)		Experimental data				
4	50	μg/ml	0.000	1.183	2.365	4.731	9.461
		% Inhibition	0.000	3.571	9.524	15.923	29.464
	60	μg/ml	0.000	1.263	2.527	5.054	10.107
		% Inhibition	0.000	2.530	5.952	13.690	26.637
	70	μg/ml	0.000	1.317	2.634	5.269	10.538
		% Inhibition	0.000	3.066	6.423	14.307	26.861
	79	μg/ml	0.000	2.567	5.134	10.269	20.538
		% Inhibition	0.000	7.153	12.993	22.774	42.920

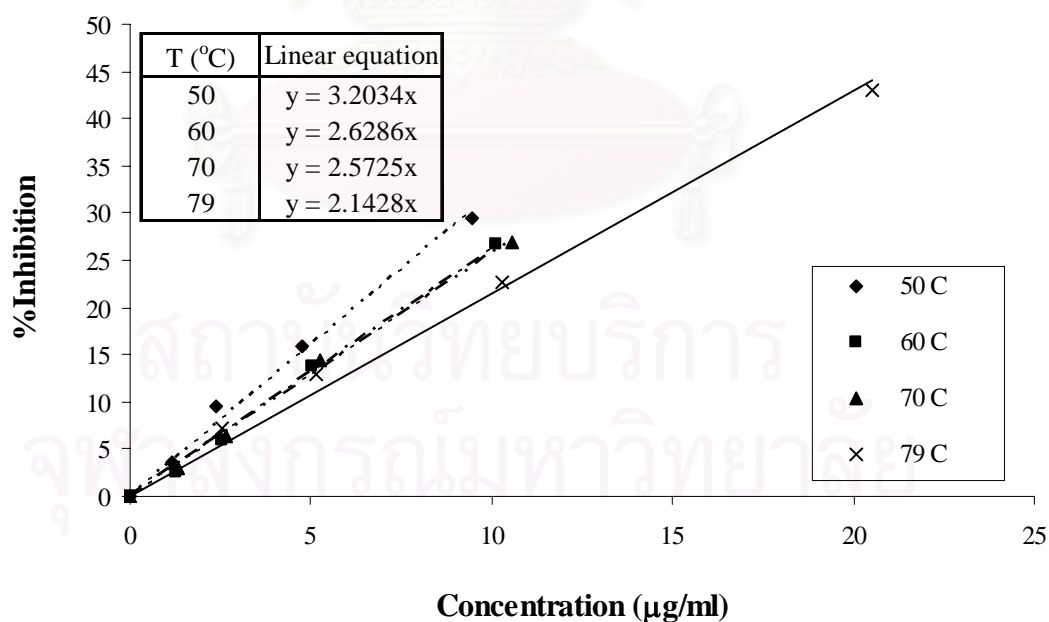**Figure B-5.5 Percent inhibition of 4 hours ethanolic extraction of yellow silk waste at various temperature, 50-79°C**

**Table B-5.8 IC$_{50}$ of SWE of 4 hour ethanolic extraction of yellow silk waste at
various temperature, 50-79$^{\text{o}}$C**

Time (h)	T ($^{\text{o}}$C)	Linear equation	IC$_{50}$ (μg/ml)
	50	y = 3.2034x	15.608
	60	y = 2.6286x	19.022
4	70	y = 2.5725x	19.436
	79	y = 2.1428x	23.334

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