APPENDIX

APPENDIX

Determination of Pigment, Sericin and Fibroin percents

The pigment extracted was recovered as powder in a rotary evaporator, followed by removing precipitated salt and a freeze-drier to obtain pigment powder. The percentage of pigment can be calculated by the following equation.

$$\% \text{ w/w} = \frac{W_P}{W_{SC}} \times 100$$

Where W_P = weight of pigment powder (g)

W_{SC} = weight of silk cocoon (g)

The weights of silk cocoon, pigments powder, and percentage of pigment are shown in Table 1.

Table 1 Weights of silk cocoon, pigments powder and percentage of pigment.

Source	Trial	Weight			
		W_{SC} (g)	Pigment powder		
			W_p (g)	% w/w	W_1 (mg/g)
Nan	1	6.003	0.145	2.42	24.2
	2	6.002	0.148	2.46	24.6
	3	6.002	0.138	2.30	23.0
	4	6.000	0.146	2.43	24.3
	Mean ± SD		0.144 ± 0.003	2.40 ± 0.06	24.0 ± 0.6

Table 1 Continued.

Source	Trial	Weight			
		W_{SC} (g)	Pigment powder		
			W_p (g)	% w/w	W_2 (mg/g)
Chiang Mai	1	6.004	0.149	2.48	24.8
	2	6.000	0.147	2.45	24.5
	3	6.000	0.143	2.38	23.8
	4	6.001	0.149	2.47	24.8
	Mean ± SD		0.147 ± 0.002	2.45 ± 0.04	24.5 ± 0.4

For example:

From **Table 3.2**, Nan (Trial 1)

$$\% \text{ w/w} = \frac{0.145}{6.003} \times 100$$

$$= 2.42\%$$

After pigment extraction, the residue from pigment extraction was degummed by alkali buffer and surfactant solution. Sericin powder was obtained after precipitation, dialysis and freeze-dry. The percentage of sericin can be calculated by the following equation.

$$\% \text{ w/w} = \frac{W_S}{W_{SC}} \times 100$$

Where W_S = weight of sericin powder (g)

W_{SC} = weight of silk cocoon (g)

The weights of silk cocoon, sericin powder, and percentage of sericin are shown in Table 2**.**

Table 2 Weights of silk cocoon, sericin powder and percentage of sericin**.**

Source	**Trial**	**Weight**			
			Sericin powder		
		W_{SC} (g)	**W_S (g)**	**% w/w**	**W_2 (mg/g)**
Nan	1	6.003	0.412	6.87	68.7
	2	6.002	0.425	7.08	70.8
	3	6.002	0.431	7.18	71.8
	4	6.000	0.357	5.95	59.5
	Mean ± SD		0.423 ± 0.008	6.77 ± 0.5	67.7 ± 4.9
Chiang Mai	1	6.004	0.506	8.4	84.3
	2	6.000	0.513	8.6	85.5
	3	6.000	0.429	7.2	71.5
	4	6.001	0.420	7.0	70.0
	Mean ± SD		0.467 ± 0.049	7.78 ± 0.7	77.8 ± 7.1

For example:

From **Table 3.3**, Nan (Trial 1)

$$\% \text{ w/w} = \frac{0.412}{6.003} \times 100$$

$$= 6.87\%$$

The fibroin powder was obtained by degumming the silk cocoon to remove a sericin. The degummed silk was dissolved in mixture solution $CaCl_2$, Ethanol and water, then purified by dialysis and freeze-dry. The percentage of fibroin can be calculated by the following equation.

$$\% \text{ w/w} = \frac{W_F}{W_{SC}} \times 100$$

Where W_F = weight of sericin powder (g)

W_{SC} = weight of silk cocoon (g)

The weights of silk cocoon, fibroin powder, and percentage of fibroin are shown in Table 3.

Table 3 Weights of silk cocoon, fibroin powder and percentage of fibroin**.**

Source	**Trial**	**Weight**			
			Fibroin powder		
		W_{SC} (g)	**W_F (g)**	**% w/w**	**W_3 (mg/g)**
Nan	1	6.003	3.485	58.1	581
	2	6.002	3.129	52.2	522
	3	6.002	3.283	54.7	547
	4	6.000	3.313	55.2	552
	Mean±SD		3.302 ± 0.146	55.0 ± 2.1	550 ± 21
Chiang Mai	1	6.004	3.756	62.6	626
	2	6.000	3.513	58.5	585
	3	6.000	3.102	55.0	550
	4	6.001	3.062	54	543
	Mean±SD		3.358 ± 0.334	58 ± 4	576 ± 33

For example:

From **Table 3.3**, Nan (Trial 1)

$$\% \text{ w/w} = \frac{3.485}{6.003} \times 100$$

$$= 58.1\%$$

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THE RELEVANCE OF THE RESEARCH WORK TO THAILAND

Silks have been gaining widely use in a variety of applications such as degradable biomaterials, biomedical and functional bio–membrane materials, cosmetics, polymer and pharmaceutical products. In Thailand, there are many silk yarn and textile handicrafts factories that annually produce large amounts of waste (from unreel silk cocoon). It can represent a significant economic and social benefit for Thailand.

Since this research aims to exhaustively separate and determine the constituents, i.e. pigments, sericin and fibroin which are present in yellow Thai silk cocoon that obtained from silkworm, *Bombyx mori,*. Having known the contents of the cocoon's constituents and their economic importance and usefulness will help support on reducing the silk waste from textile handicrafts factories that are normally exploited undercostedly. Therefore, the utilization of silk and its related parts can be of great benefits economically and socially.

EFFECT OF SOLVENT SYSTEMS ON REFLUX EXTRACTION OF PIGMENTS IN YELLOW *THAI SILK COCOON*

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Abstract

Pigments in yellow Thai silk cocoons (var. Nangnoi Srisaket) were exhaustively extracted by optimizing the extraction conditions. These pigments are highly efficient antioxidants and have been used in various pharmaceutical and cosmeceutical applications. The optimization was initially started by selecting a better solvent from methanol and ethanol. The cocoons were refluxed in either methanol and ethanol by varying their contents between 60–100%. The polarity of the selected solvent was reduced with either hexane or acetone at 9/1 and 19/1 volume ratios to evaluate its polarity effect. Finally, the solvent strength was modified by adding acid or alkaline or salt. The amount of pigment extracted was monitored by spectrophotometry. It was found that 80% ethanol was a better extraction solvent without the addition of both hexane and acetone. The extraction could also be carried out more efficiently under alkaline condition by adding basic salt but the longer period of extraction time caused the decomposition of the pigment. Thus it can be concluded that 0.80 M CH_3COONa in 80% ethanol (v/v) was suitable extraction solvent with 30 min. of extraction time at 80°C the total amount of pigments in the silk cocoon was obtained as 22.3 $\pm$ 0.3 mg/g silk cocoon by repeating reflux extraction 4 times.

Keywords: Pigments, Silk cocoon

Introduction

Silk is a well-known natural fiber produced by silkworm which has been used traditionally in the form of thread in textiles for thousands of years. It exhibits qualities of strength, elasticity, softness, absorbency and affinity for dyes [1]. Raw silk fiber obtained from *Bombyx mori* silkworms consists of major proteins, namely fibroin (70–80%) and sericin (20–30%) as well as other impurities such as wax (0.4–0.8%), moisture (10–11%), carbohydrates (1.2–1.6%), inorganic matters (0.7%) and pigments (0.2%) . The silk sericin envelops the fibroin fiber with successive sticky layers to help in the formation of a cocoon. It is the silk sericin and other impurities that mask the luster of the silk

fibroin and cause hardness and coarseness of raw-silk fibers [2].

The pigments have variations in their nature depending on the types of silk and their presence in the sericin layer causes the variation of colour such as yellow, yellowish, green, brown and golden yellow. This colour is not permanent and can be washed away with the sericin during the degumming process [3]. The yellow silk cocoon can be found in Thailand, such as those varieties of Nangnoi Srisaket, Nangnoi Sakon Nakhon, Udonthani and Ubon Ratchathani 60-35 or Dokbua. In previous studies, the major carotenoid in yellow silk was reported to be lutein (β,ε-carotene-3,30-diol) about 80–90% of total carotenoids, whereas the β-carotene and α-carotene are minor components. Lutein itself has been reported as being an excellent agent in protecting against vision loss and reducing the risk of chronic disease, such as heart disease, cancer [4-5]. Recently, researchers have investigated the yellow Nangnoi Srisaket silk cocoon and found that it contains a significant amount of pigments. These pigments are mostly associated with carotenoids and flavonoids and reported as being highly efficient antioxidants. Therefore, they both have been used in various pharmaceutical and cosmeceutical application [5-6].

There have been numerous reports related to extraction and identification of pigments from silk cocoon. Tamura *et al*. [4] identified three flavonoid typed 5- glucosides including quercetin 5-glucoside, quercetin 5,4′ diglucoside, and quercetin 5,7,4′-triglucoside from the yellow-green cocoon shell of the "Multi-Bi" silkworm of *Bombyx mori*. Pigments were extracted from the cocoon shell by MeOH–H_2O (2:1, v/v) at 60°C for 1 h. then detected and identified by HPLC, UV–Vis, FT–IR, and NMR. Prommuaka *et al*. [6] studied the effect of solvent systems on extraction efficiency of carotenoids and flavonoids from yellow Thai silk waste. Different methods for extractions of these pigments were carried out using ethanol and subcritical water. For extraction of carotenoids, the ethanol was suitable extraction solvent and the amount of carotenoids increased with increasing temperature (50–79°C) and extraction time (2–12 h). For flavonoids, subcritical water extraction was suitable at 120°C for 10 min. But the amount of flavonoids decreased with increasing subcritical water temperature and extraction time due to decomposition at such conditions. Hirayama *et al*. [7] extracted the yellow pigments from the cocoon shell sample with MeOH–H_2O (2:1, v/v) at 60°C for 2 h. The extracted was pigment filtered and concentrated under reduced pressure, after which H_2O was added. The aqueous solution was applied to a solid-phase extraction. Two flavonoids containing the L-proline moiety, 6-C-[(2S,5S)-prolin-5-yl] quercetin and 6-C-[(2S,5R)-prolin-5-yl] quercetin were then investigated by spectroscopic methods.

Although a number of recent studies have been conducted to extract and identify different constituents of silk pigments [4-7], the purposes of the present study are to optimize the condition

for extraction of pigments using different solvent systems in order to obtain a better yield of pigments. The total amount of pigments in Nangnoi Srisaket silk cocoon will be determined. This study will lead to quantification of the pigments, fibroin and sericin in order to correlate their contents in the silk cocoons.

Materials and Methods

Materials

Raw yellow silk cocoon sample of a polyvoltinerace (called locally as Nangnoi) of Thai silkworms, *Bombyx mori* were obtained from The Queen Sirikit Sericulture Centre, Nan Province, Thailand. Raw sample was stored at room temperature until analysis.

All reagents used for extraction were of analytical grade.

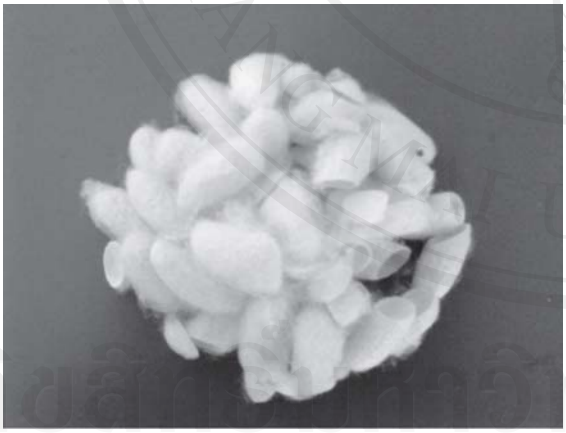

Figure 1 Nangnoi Srisaket silk cocoon of *Bombyx mori* silkworms obtaining in Thailand.

Factors affecting pigment extraction

a. Effect of methanol and ethanol

The silk cocoon was cut into pieces with an average length of 10 mm. Approximately 3.0 g of dried silk cocoon was placed in a 250 mL round bottom flask and was then extracted with 100 mL of various concentrations of aqueous EtOH solution (60%, 70%, 80% and 90% v/v) and aqueous MeOH solution (60%, 70%, 80%, 90% and 100% v/v) by heat reflux extraction at 80°C for 2 h. Then, the sample was immediately cooled to room temperature. Each extracted solution was finally filtered. For EtOH extract, 2 mL was pipetted, then diluted to 5 mL in a volumetric flask before measuring the absorbance, whereas the absorbances of MeOH extracts were measured directly. All visible absorption measurement was done spectrophotometrically (Jenway 6400, USA).

In the following experiments, factors such as solvent polarity, acid-base condition and ionic strength of the extracting solvent were studied using the same extraction procedure, i.e. reflux extraction of 1.5 g of yellow silk cocoon with 50 mL of each conditioning solvent under study at 80°C for 30 min. The absorbances of the extracts were then measured spectrophotometrically in the same manner as done with the study of the effect of alcohol concentration. The extraction efficiency was evaluated. Therefore, only the changes of the condition are described below.

b. Effect of solvent polarity

The polarity of the appropriate solvent for pigment extraction concluded (from a) was modified by mixing with either hexane or acetone to obtain the solutions with the volume ratios of 19/1 and 9/1 (80% v/v EtOH/hexane or acetone).

c. Effect of acidity/alkalinity

The extraction condition was modified by the addition of either acetic acid or sodium hydroxide at different concentrations.

A set of acidic treatment was done by extracting 1.5 g of yellow silk cocoon with mixed solvents of 80% EtOH and acetic acid at various mixing volume ratios (100:0, 90:10, 80:20, 70:30 and 60:40).

As for alkaline treatment, sodium hydroxide was used in place of acetic acid and the experimental procedure was the same as done with acetic acid. The sodium hydroxide was also varied in its concentration to be 0.01 M, 0.05 M and 0.10 M in 80% EtOH.

d. Effect of ionic strength

The ionic strength of the solvent was adjusted using different types of salt, namely KCl, $CaCl_2$ and CH_3COONa in 80% EtOH. The concentration of each salt was maintained at 0.01 M.

e. Effect of CH_3COONa concentration

The effect of CH_3COONa concentrations was studied by varying the concentrations to be 0.01, 0.02, 0.10, 0.20, 0.40 and 0.80 M in 80% EtOH.

Determination of the total pigment

The pigment was removed from the silk cocoon sample by repeating reflux extraction. 3.0 g of the silk cocoon sample was extracted with 100 mL of suitable solvent. The extract cocoon was then re-extracted in the reactor under the same condition (total of 4 times). The crude pigment extract was processed by using a rotary evaporator at 40°C. and a freeze-drier to obtain pigment powder. The percentage of pigment in the silk cocoon was determined gravimetrically.

Results

Effect of methanol and ethanol

Generally, MeOH or EtOH solutions containing some water, particularly those ranging from 40% to 80% MeOH or EtOH, are more efficient in extracting polyphenolic compounds than pure water and absolute MeOH or EtOH [8]. In this study, the investigation of using different MeOH (and EtOH) concentrations on the extraction of pigments also provided the same conclusion. From Figures 2 and 3, reveal that 80 % v/v EtOH is the most effective extracting solvent observed from the maximum absorbance value compared with other solvents. Therefore, 80% v/v EtOH aqueous solution was chosen as the extractant for silk cocoon sample analysis.

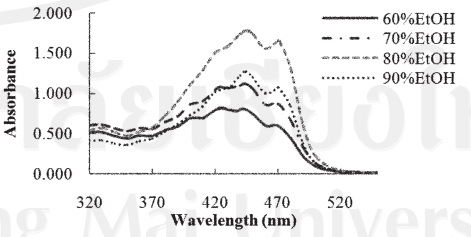

Figure 2 Effect of EtOH concentrations on the extraction of pigments.

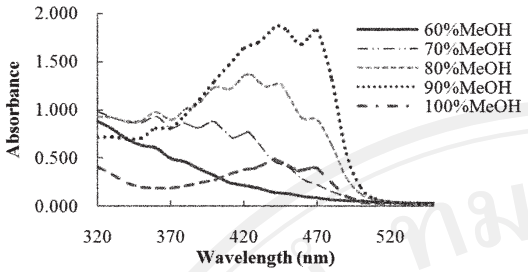

Figure 3 Effect of MeOH concentrations on the extraction of pigments.

Effect of solvent polarity

Result of modifying solvent polarity by mixing 80% EtOH with hexane and acetone at volume ratios of 19/1 and 9/1were observed by measuring absorbances at 424, 444 and 474 nm. It is seen in Table 1, that in all cases of having additional solvent in the system, the extraction efficiency decreases that may be due to the reduction of solvent strength.

Table 1 Effect of solvents polarity on pigment extraction.

Solvent	Ratio	Absorbance		
		424 nm	444 nm	474 nm
80%EtOH	-	1.28 ± 0.08	1.68 ± 0.08	1.30 ± 0.08
80%EtOH:acetone	9/1	1.23 ± 0.06	1.64 ± 0.07	1.29 ± 0.06
80%EtOH:hexane	9/1	1.21 ± 0.04	1.08 ± 0.05	0.81 ± 0.04
80%EtOH:acetone	19/1	1.21 ± 0.02	1.60 ± 0.03	1.26 ± 0.02
80%EtOH:hexane	19/1	0.83 ± 0.01	1.61 ± 0.03	1.24 ± 0.01

***Effect of* acidity/alkalinity and salt**

As silk fibers consist of acidic group (-COOH) and basic groups ($-NH_2$) in the molecule, therefore these polar functional groups tend to interact with the polar part of the pigment molecules. However, alteration of the pH of the solution often affect the polarity of the attracted species by breaking loose of the pigments. So the extraction result using acetic acid at different concentrations is shown in Figure 4.

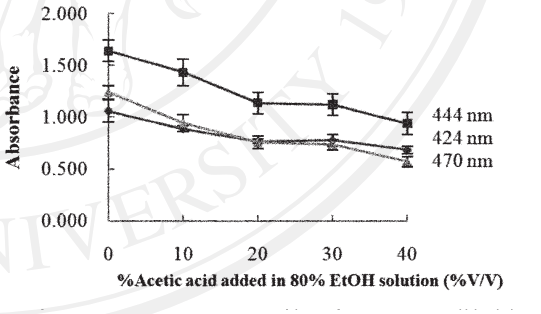

Figure 4 Effect of the percentage of acetic acid added in 80% EtOH solution (%v/v) on pigments extraction.

It is observed that the presence of acetic acid affects the extraction of pigment revealed from the decrease of the absorbance with increasing content of % acetic acid. But in the case of NaOH addition, the extraction became more efficient with the increase of NaOH concentrations (Table 2).

Table 2 Effect of sodium hydroxide concentrations in **80% v/v EtOH** on **pigments extraction.**

[NaOH] in 80%v/v EtOH (Mol/L)	Absorbance		
	424 nm	444 nm	474 nm
0.00	1.28 ± 0.08	1.68 ± 0.08	1.30 ± 0.08
0.01	1.42 ± 0.03	1.70 ± 0.03	1.55 ± 0.02
0.05	1.48 ± 0.02	1.77 ± 0.02	1.68 ± 0.01
0.10	1.48 ± 0.01	1.78 ± 0.01	1.76 ± 0.01

This indicates that the characteristic of the pigment structures should be acidic in their nature. Since it has been reported that the major pigment present in the yellow silk is lutein (80% of total carotenoids) [9]. Its structure consists of OH groups that can dissociate showing its acid property (Figure 5). So the pigments will better react with the base and can be extracted in a greater extent.

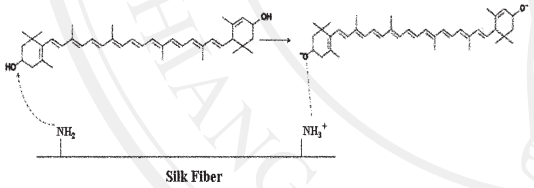

Figure 5 The ionic interaction between lutein and silk fibroin.

However, for alkaline condition, if high concentration of NaOH is used, it will destroy the pigments and cause the degradation of the cocoon. Therefore the 0.01 M NaOH was selected for extraction. Meanwhile, the effect of salts on the efficiency of pigments extraction using three types of salt; KCl, $CaCl_2$ and CH_3COONa at 1.0 M were studied.

Variation in the absorbance values of the pigment extracted when various kinds of salts were added is shown in Table 3.

Table 3 Effect of salt addition on pigments extraction.

80%v/v EtOH	Absorbance		
	424 nm	444 nm	474 nm
Without salt	1.28 ± 0.08	1.68 ± 0.08	1.30 ± 0.08
1% of 1.0 M KCl added	1.09 ± 0.02	1.40 ± 0.02	1.08 ± 0.02
1% of 1.0 M $CaCl_2$ added	1.23 ± 0.01	1.47 ± 0.02	1.15 ± 0.02
1%of 1.0 M CH_3COONa added	1.27 ± 0.01	1.73 ± 0.01	1.40 ± 0.02

It was found that the absorbances decreased with the addition of neutral salts (KCl and $CaCl_2$), but increased slightly with the addition of basic salts (CH_3COONa) which produced the same result as with the addition of NaOH. This observation confirms that the optimal condition for pigment extraction should be alkaline medium. Moreover, when the effect of ionic strength is considered, the $CaCl_2$ solution which has higher ionic strength compared to NaCl solution showed

only a slightly better extracting power for pigments. This is because when the pigment is removed from the silk fiber through the acid-base reaction, the pigment molecules will be surrounded by the salt ions. The more ions are present in the solution, the better the hindrance effect the ions impose to the pigments. Thus it prevents the pigments to be readsorbed to the fiber. Therefore the CH_3COONa was chosen for studying the effect of concentration (Table 4).

Table 4 Effect of various concentration of CH_3COONa on solvent for extraction of pigment.

[CH_3COONa] in 80%v/v EtOH (Mol/L)	Absorbance		
	424 nm	444 nm	474 nm
0.00	1.28 ± 0.08	1.68 ± 0.08	1.30 ± 0.08
0.01	1.33 ± 0.01	1.78 ± 0.01	1.42 ± 0.02
0.02	1.38 ± 0.03	1.84 ± 0.02	1.48 ± 0.02
0.10	1.43 ± 0.01	1.91 ± 0.01	1.56 ± 0.01
0.20	1.45 ± 0.04	1.94 ± 0.03	1.60 ± 0.03
0.40	1.48 ± 0.02	1.96 ± 0.02	1.63 ± 0.02
0.80	1.50 ± 0.03	1.99 ± 0.02	1.66 ± 0.02

It was also observed that the extraction yield of pigment was increased with the increase of CH_3COONa concentration. Upon making a comparison between 0.8 M CH_3COONa and 0.01 M NaOH, it was found that 0.8 M CH_3COONa was more effective as a solvent for pigment extraction than 0.01 M NaOH. Thus, 0.8 M CH_3COONa in 80 %v/v EtOH was selected as a suitable solvent for pigments extraction.

Determination of total pigments content

After the pigments were extracted repeatedly using 0.8 M CH_3COONa in 80% EtOH (v/v) at 80°C for 30 min, the extract was then recovered as powder in a rotary evaporator, followed by removing precipitated salt. It was found that the yield of the extracted pigment powder was 22.3 $\pm$ 0.3 mg/g silk cocoon.

Conclusions and Discussion

This study demonstrated that the yellow pigments of Thai silk cocoon can be effectively extracted through refluxing with 0.8 M CH_3COONa in 80% EtOH at 80°C for 30 min. The total amount of pigments in the silk cocoon was found to be 22.3 $\pm$ 0.3 mg/g of dry weight by repeating reflux extraction 4 times.

This modifying method can also be applied to the extraction of pigment in other silk cocoon samples.

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