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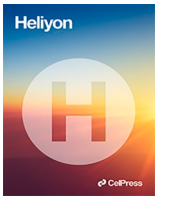

Storage stability and antioxidant activities of lutein extracted from yellow silk cocoons (*Bombyx mori*) in Thailand

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ABSTRACT

This study aimed to determine how different forms of lutein found in nature affected their thermal stability, degradation, and antioxidant activities. The findings show that commercial lutein (CL) degraded faster than silk luteins (SLs) at ≤ 4 °C. The two-stage first-order kinetics of thermal degradation showed that *Ea* for SLs was 4.6–9.5 times higher than CL. However, at ≥ 25 °C, both the CL and SLs degraded rapidly within one month. SLs had half-life at 4 °C from 10 to 104 wks. FTIR and HRMS analysis revealed that their oxidation products were similar ($C_{18}H_{26}O_2$: 297 m/z). Based on IC_{50}, antioxidant activities of SLs were superior to CL. The stability and antioxidant capacity of lutein may be influenced by its naturally occurring forms. The naturally occurring forms and unpurified state of lutein can affect its stability and antioxidant activity, which must be considered when storing lutein at different temperatures.

1. Introduction

Lutein (β,ε-carotene-3,3'-diol) plays an important role throughout our lives, particularly in the functions of eye and brain tissue [1]. Several cerebral processes, including antioxidant activity, cognitive efficiency, and memory capacity, are promoted by lutein in brain tissue [2]. By its antioxidant property, lutein functions as a light filter in the retina to reduce oxidative damage caused by high-intensity "blue light." [3]. Although lutein can be obtained from yellow-pigmented animals, foods, and plants such as laying hens, egg yolks, spinach, kale, and algae [4–6], lutein intakes may not be adequate to meet population needs for optimal health [7] and are insufficient to prevent eye diseases, particularly cataracts and age-related macular degeneration (AMD). The average daily consumption of lutein in the United States and Europe is only 2.45 mg per day [8], whereas the suggested daily intake is around 6 mg/day to reduce the risk of AMD by 57% [7]. Currently, lutein is available as a dietary supplement with a recommended daily intake of 6–10 mg [9]. Due to lutein's instability and chemical alterations that occur during food preparation, its use in the food industry is constrained and subjected to research for shelf-life extension [10]. Because of its antioxidant activity, lutein degrades rapidly when stored at elevated temperatures, which is crucial during transport and storage in tropical climates. Under high temperatures, lutein degrades into low molecular weight molecules [11], and the rate of lutein breakdown accelerates as the temperature increases [12]. In general, lutein contains double bonds in the trans form, and when exposed to heat, a conformational change to the less stable cis isomer happens [13]. Lutein isomerization and decomposition occur when the food matrix is subjected to heat treatment [14–18]. A previous study by Ref. [17]

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concluded that lutein decomposition under various heating conditions followed second-order kinetics. In their investigation, lutein loss during the storage period was minimal before the temperature reached 40 °C. Significant degradation was found between 50 °C and 60 °C. However, some commercial lutein products purchased locally in Thailand decayed much faster than the expiration date indicated on the label, causing problem for both consumers and producers.

The yellow silk cocoon of the silkworm (*Bombyx mori*) can be an alternative source of high-quality lutein. Approximately 90% of lutein and its isomers can be recovered directly from degummed yellow silk cocoons at a concentration of 60 mg/100 g dry weight (dry basis, %db). It is also a source of lutein that is considered organic and proven to be protein-binding lutein [19], which is distinct from the free lutein or lutein ester form found in fruits, vegetables, and flowers [20]. In both *in vitro* and *in vivo* studies, silk lutein has shown high efficacy. Extracts of lutein from silk cocoons contained a combination of lutein and fatty acids, which may boost its effectiveness in animal models [21]. In numerous Asian nations, silkworms and silk cocoons are considered delicacies. Additionally, according to the FAO, silkworms are among the top 5 edible insects. Subsequently, it is noteworthy that yellow silk cocoons will likely emerge on the food market as a new source of lutein for dietary supplements. Since lutein is commonly used as a dietary supplement, understanding its stability under storage conditions is vital. To date, no studies have evaluated the impact of various lutein sources, hence, natural existing forms, on their stability, degradation kinetics, and oxidation products. Therefore, the goals of this work were 1) to investigate the stability of lutein from two distinct sources: yellow silk cocoons (*Bombyx mori*) (protein-binding lutein) and commercial lutein (free lutein) from marigold while stored under identical conditions, and 2) to compare their antioxidant efficacy and identify their oxidation products using Fourier transform infrared spectroscopy (FTIR) and high-resolution mass spectrometry (HRMS). The results of this study contribute to our understanding of the important role of lutein sources in its stability at various storage temperatures.

2. Materials and methods

2.1. Materials

The 5 varieties of Thai native yellow silk cocoons (Nang Noi Srisaket-1 (NN), Nang Sew (NS), Nang Tui (NT), Nang Lai (NL), and Samrong (SR)) were acquired from Sericulture farms and Queen Sirikit Sericulture Centers located in the Thai provinces of Nakhon Ratchasima, Udon Thani, Chaiyaphum, Buriram, and Khon Kaen. Butylated hydroxyanisole (BHA), Tocopherol, and lutein analytical standards were purchased from Sigma-Aldrich Co., Ltd. (MO, USA.). Carlo Erba Reagents supplied methanol, acetonitrile, ethanol, ethyl acetate, Folin-Ciocalteu reagent, and n-hexane of high-performance liquid chromatography (HPLC) grade (Arese, Italy). Commercial lutein (95% xanthophylls, extracted from Marigold flower) was purchased from TTK Science Co., Ltd (Bangkok, Thailand).

2.2. Silk lutein extraction

The lutein extraction procedure was based on previous studies by Refs. [15,22–25] with some modifications. Approximately 1 g of Thai yellow silk cocoons from each variety were soaked in distilled water at a ratio of 1:30 before being degummed at 121 °C for 15 min. Filtration was used to separate the degummed solutions containing sericin and the degummed silk cocoons. Lutein was extracted from each part three times with 30 mL of an organic mixture solution of hexane, ethyl alcohol, and ethyl acetate (3:2:1 by v/v/v) and 0.1% BHA (w/v). The collected extraction solutions were partitioned into a non-aqueous phase and an aqueous phase by adding 100 mL of 10% NaCl (w/v) to the organic mixture solution [24]. This partitioning step was repeated with the addition of the organic mixture solution until the aqueous phase of the extraction solution became colorless. To obtain the dried extract, the non-aqueous or organic phase was collected and concentrated to dryness in an amber glass by vacuum evaporation at 35 °C. After drying, the lutein extract was transferred to screw-capped amber vials, flushed with nitrogen gas, and stored at −80 °C for further analysis.

2.3. Determination of lutein content

All analyses were performed on an Agilent HP 1120 Compact LC HPLC system with a photodiode array detector. Data analysis was performed using Plumbagin software (Agilent Technologies, Waldbronn, Germany). The separation was performed on a LiChrospher® 100RP-18C column (250 mm × 4.60 mm internal diameter; 5 μm particle size) (Merck, Germany) [26] maintained at 20 °C with the mobile phase consisting of a mixture of solvent A (acetonitrile:methanol, 9:1 v/v) and solvent B (ethyl acetate) at a flow rate of 0.5 mL/min [27]. Elution was carried out with a gradient program: 20% B for 15 min, 20–50% B for 15–20 min, 50-20% B for 20–25 min, and maintained at 20% B for 25–40 min. Each sample was dissolved in absolute ethanol and filtered through a 0.45 μm diameter syringe filter into a 5.00 mL amber vial. The injection volume was 10.00 μL. The detection wavelength was set at 445 nm [28].

2.4. Stability test of lutein

The effect of storage temperature and time on the degradation of lutein in CL and SLs was investigated. To obtain the SL extract stock solution, the dried SL extract was redissolved in absolute ethanol, and the final volume was adjusted to 5 mL. A 500 μL aliquot of the extract stock solution was then pipetted into a 4 mL vial and dried with nitrogen gas flow. To prevent lutein deterioration in the presence of oxygen, samples were additionally flushed with nitrogen before being hermetically packed. Each sample was separated and incubated independently in the dark at −80, −20, 4, 25, and 40 °C for 0, 1, 2, 4, 7, 19, and 48 weeks. HPLC was used throughout the study to determine the concentration of lutein at each selected week of the experiment [21,28,29]. The lutein retention was determined by comparing the weekly lutein concentration to the initial lutein concentration.

2.5. Degradation kinetic analysis

A kinetic model was fitted to a plot of natural-log lutein concentration or the inverse of lutein concentration vs storage time to get the degradation rate constants (k). The kinetics of experimental data were evaluated using the first and second-order [26] equations (1) and (2):

$$In(C / C_0) = -kt \tag{1}$$

$$1 / C = 1/C_0 + kt \tag{2}$$

where C, C_0 and t are the remaining concentration of lutein after storage under each storage condition, the initial concentration of lutein, and the storage time, respectively. At each storage temperature, the half-life ($t_{\frac{1}{2}}$) was calculated using the rate constant of the degradation kinetic. The half-life for the concentrations in silk lutein extract to degrade to 50% of their initial concentrations was calculated by equation (3):

$$t_{\frac{1}{2}} = 1 \Big/ [k(C_0)] \tag{3}$$

where k is the rate constant of lutein degradation at each storage temperature. To examine the effect of temperature on the degradation rate of lutein, the activation energy (Ea) of thermal destruction was calculated using equation (4):

$$k = k_0 \left[\frac{1}{e^{Ea/RT}}\right] \tag{4}$$

where Ea is the activation energy (kJ/mol), k_0 is the frequency factor or pre-exponential factor calculated from the y-intercept of the linear plot of ln k vs. $\frac{1}{T}$ of lutein samples from each variety, R is the gas constant (8.314 J/mol/K), and T is the absolute temperature of storage temperature (K).

2.6. Molecular structure and chemical bonding of lutein during storage

FTIR and HRMS were used to further examine and identify the molecular structure, functional groups, and chemical bonds of lutein during degradation. The CL, SLs, and HPLC-purified SLs were investigated and subsequently stored at −80 °C and 25 °C for 1 and 2 weeks, respectively [21,28,29].

2.6.1. Fourier transform infrared spectroscopy (FTIR)

Infrared spectra of lutein in CL, SLs, and HPLC-purified SLs samples were recorded on a Tensor 27 FTIR spectrometer (Bruker Optics, Ettlingen, Germany). Samples were collected at random each week and redissolved in absolute ethanol to produce a solution with a concentration of 20 g/mL. The solutions were finally filtered through a 13 mm diameter syringe filter with a 0.45 μm pore size into a 2 mL amber vial. 40 μL of lutein solution was dropped on a platinum attenuated total reflectance (ATR) surface and left to air dry for 5 min. The infrared spectra of functional groups in the samples were acquired at 25 °C in the spectral region of 3500–400 cm^{-1} [30].

2.6.2. High-resolution mass spectrometry (HRMS)

HRMS (Bruker, micrOTOF-QIII, Bruker Optics, Ettlingen, Germany) with nano-electrospray ionization (ESI) source and flame ionization detector was used. CL, SLs, and HPLC-purified SLs samples were prepared in the same manner as in 2.6.1, with 100.00 μL/min injected into the equipment. The mass spectra were collected in positive ionization modes over a 50–650 m/z scanning range. The following acquisition parameters were used: 4.5 kV capillary voltage, −500 V end plate offset, 100.0 Vpp collision cell RF, 0.3 bar nebulizer pressure, 180 °C dry heater, and 4.00 L/min dry gas flow rate [26]. The lutein (M^++Na^+) signal ions were recorded and compared to the HRMS mass spectra library.

2.7. Antioxidant activities of silk lutein extract

2.7.1. ABTS radical-scavenging activity

The antioxidant activity of yellow SLs was determined using a modified version of the decolorization assay of the ABTS radical mono cation (ABTS·$^+$) as described by Ref. [31]. The ABTS radical mono cation (ABTS·$^+$) solution was prepared by the reaction of 7 mM ABTS·$^+$ with 2.45 mM potassium persulfate in the dark at room temperature for 12–16 h. The ABTS solution was then diluted with ethanol to achieve an absorbance of 0.70 ± 0.02 at 734 nm and equilibrated at 30 °C. For the reaction, 10 μL of the extract was mixed at room temperature with 1 mL of ABTS radical cation (ABTS·$^+$) solution. The absorbance at 753 nm of each sample was immediately measured using a UV–Vis spectrophotometer (Amersham Biosciences, Ultrospec 6300 pro, Sweden). Various concentrations of Trolox standard solution were used to generate a standard curve. The control was ABTS·$^+$ solution, while the blank was distilled water. We determined the IC_{50} values of SLs and compared them to those of BHA and Tocopherol. The calculation for the inhibition of ABTS radicals was as follows: equation (5):

Table 1
Results of lutein content in yellow silk (*Bombyx mori*) cocoons analyzed by HPLC.

Strains	Lutein content (mg/100 g of dried silk cocoons)			%Yield
	Degumming solution ± SD	Degummed cocoons ± SD	Total of lutein ±SD	
Nang Noi Srisaket-1	17.687 ± 1.276	24.750 ± 0.085	42.437^{c} ±1.311	0.042
Nang Tui	12.163 ± 0.491	25.739 ± 1.401	37.902 ^{d} ± 1.494	0.038
Nang Sew	18.452 ± 1.030	22.927 ± 0.777	41.379^{c} ±1.618	0.041
Nang Lai	18.770 ± 0.969	27.893 ± 0.894	46.662 ^{b} ± 1.825	0.047
Samrong	22.956 ± 1.075	27.444 ± 0.833	50.400^{a} ±1.660	0.050

Note: SD: standard deviation, $n = 3$.
$^{a, b, c, d}$ Data are significant difference according to SPSS at $p < 0.05$.

$$Scavenging\ activity\ (\%) = \left[\frac{(Control\ OD - Sample\ OD)}{Control\ OD}\right] x\ 100 \tag{5}$$

where *OD* is optical density.

2.7.2. DPPH radical-scavenging assay

The *in vitro* DPPH radical-scavenging activities of SLs and CL were evaluated and compared by modifying the methods of [32,33]. Various concentrations (50, 100, and 200 ppm) of the sample extracts were mixed with 2 mL of a $6x10^{-5}$ M methanolic solution of DPPH and incubated at room temperature for 60 min. The decrease in absorbance of DPPH was measured at 517 nm. Methanol was used as a blank solution. BHA and tocopherol were used as references. All measurements were performed in triplicate. IC_{50} values of all samples were determined. The percentage inhibition of the DPPH radicals by SLs was calculated using the following equation (6) [34]:

$$\%inhibition = \left[\frac{(A_{C(0)} - A_{C(t)})}{A_{C(0)}}\right] x\ 100 \tag{6}$$

where $A_{C(0)}$ is the absorbance of the control at t = 0 min and $A_{C(t)}$ is the absorbance of the antioxidant at t = 1 h.

2.7.3. Ferric reducing antioxidant power (FRAP) assay

The ferric-reducing activities of the SL extracts were determined using a modified version of the [35] technique. Fresh FRAP reagent was made by combining 25 mL of 300 mmol/L acetate buffer, 2.5 mL of 10 mmol/L 2,4,6-tripyridyl-s-triazine (TPTZ) in 40 mmol/L HCl, and 2.5 mL of 20 mmol/L $FeCl_3\ 6H_2O$. The freshly mixed compound was incubated in a water bath at 37 °C for 5 min, and the absorbance was measured at 593 nm. After adding 10 μL of the test sample to 90 μL of distilled water, 900 μL of FRAP reagent was added. The absorbance was measured both immediately and 4 min afterward. The standard curve was developed using ferrous sulfate as the standard in the concentration range of 25–100 μL/mL. The reducing power and IC_{50} values of lutein samples were compared with those of BHA and tocopherol. The FRAP value (mol/L) was calculated using equation (7) [36]:

$$FRAP\ value = \left[\left(\frac{Sample\ OD}{Control\ OD}\right)\right] x\ Fe^{2+} standard\ concentration\ (\mu molL) \tag{7}$$

where *OD* is optical density.

2.7.4. Determination of total phenolics

The total phenolic contents of CL and the SLs were estimated using the Folin-Ciocalteau reagent as described by Ref. [37]. SLs that had been previously dried under N_2 were dissolved in ethanol and adjusted to 5 mL 10 μL of dissolved lutein was diluted with 10 μL deionized water (DI). The Folin-Ciocalteau reagent and DI water were added at 100 and 1580 μL, respectively. After 5 min, 300 μL of 20% $NaCO_3$ was added to the solutions. The absorbance was measured after 120 min at 765 nm. The total phenolic contents were calculated using the calibration curve constructed from the absorbance of gallic acid concentrations ranging from 50 to 800 μg/mL.

2.8. Statistical analysis

Data were expressed as means±standard deviations (SD) of three replicates and then analyzed by SPSS Version 16. Duncan's New Multiple-Range (DMRT) test was used to determine the differences among the means. Values with *p*-values less than 0.05 were significant.

3. Results and discussions

3.1. Determination of lutein content

Table 1 shows the contents of lutein in five varieties of Thai native silk (*Bombyx mori*) cocoons were around 0.04%db, which is in

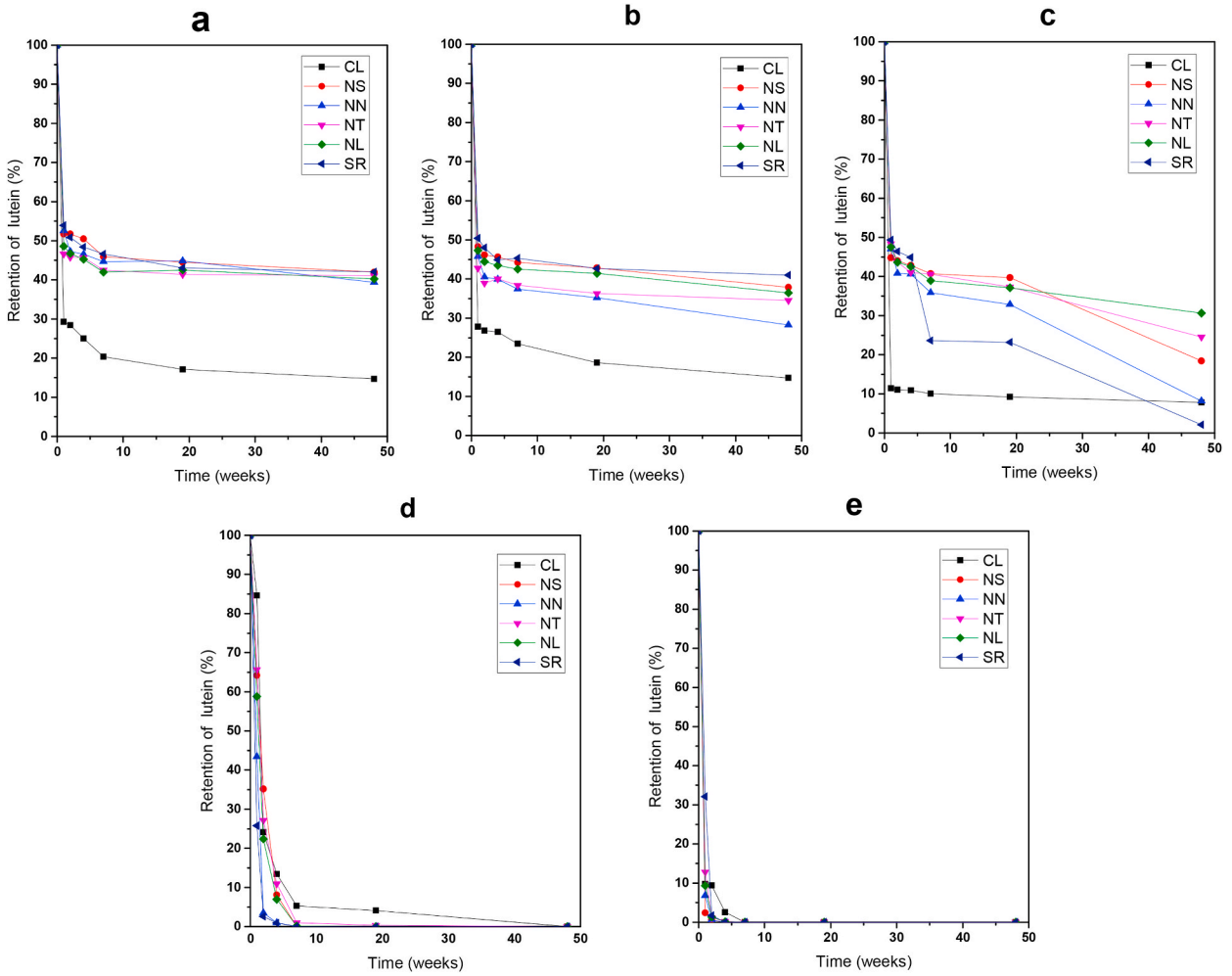

Fig. 1. Retention of Commercial lutein (CL), Nang Sew (NS), Nang Noi (NN), Nang Tui (NT), Nang Lai (NL), and Samrong (SR) lutein and time of storage at −80 °C (a), −20 °C (b), 4 °C (c), 25 °C (d), and 40 °C (e).

the range of lutein found in marigold petals (0.017–0.57%db) [27]. A few silk strains can have lutein up to 0.1%db depending on the high intensity of yellow. Silk cocoons with an orange-yellow color similar to duck egg yolk usually have the highest amount of lutein. This information suggests that cocoons of Thai natural silk (*Bombyx mori*) can be exploited as a novel source of lutein for dietary supplements and nutraceutical products. Lutein in yellow silk cocoons is considered an unwanted co-product and needs to be removed during silk degumming and reeling steps along with sericin. Extraction of lutein from silk materials will be beneficial to the environment and provide a sustainable income to sericulture farmers.

3.2. *Stability test of lutein*

Fig. 1 shows the retention of lutein in CL and SLs versus time at various temperatures (−80 °C–40 °C). Fig. 2 depicts typical HPLC chromatograms of lutein samples in this study. Lutein rapidly decreased at 0–1 week due to thermal degradation and eventually reached a plateau. A few studies have previously observed this pattern and explained the rapid degradation of lutein, particularly during the first week. Carotenoids-5,6-epoxide and highly reactive alkoxy radicals formed and accumulated during the lutein extraction process could contribute to lutein depletion via lipid autooxidation [38]. After the highly reactive alkoxy radical species have been scavenged, lutein continues to react to low-energy radicals at a much slower rate, as shown in the second stage [39]. Even at low concentrations, free lutein (FL) from marigold was reported to have prooxidant activity and induced lipid oxidation of purified corn triacylglyceride (TAG) more than lutein dimyristate (LD). However, as the concentrations of FL and LD increased, so did the lipid oxidation of TAG [40]. In nature, lutein coexists with phenolic compounds and flavonoids. Interactions of flavonoids and carotenoids in heterogeneous systems may play an important role in antioxidant synergism and antagonism, influencing the kinetic rate of lipid autooxidation during storage [41]. Furthermore, during the extraction process, lutein was subjected to environmental stresses such as mechanical energy, heat, oxygen, and light for a long time, resulting in a more favorable environment for the formation of free radicals,

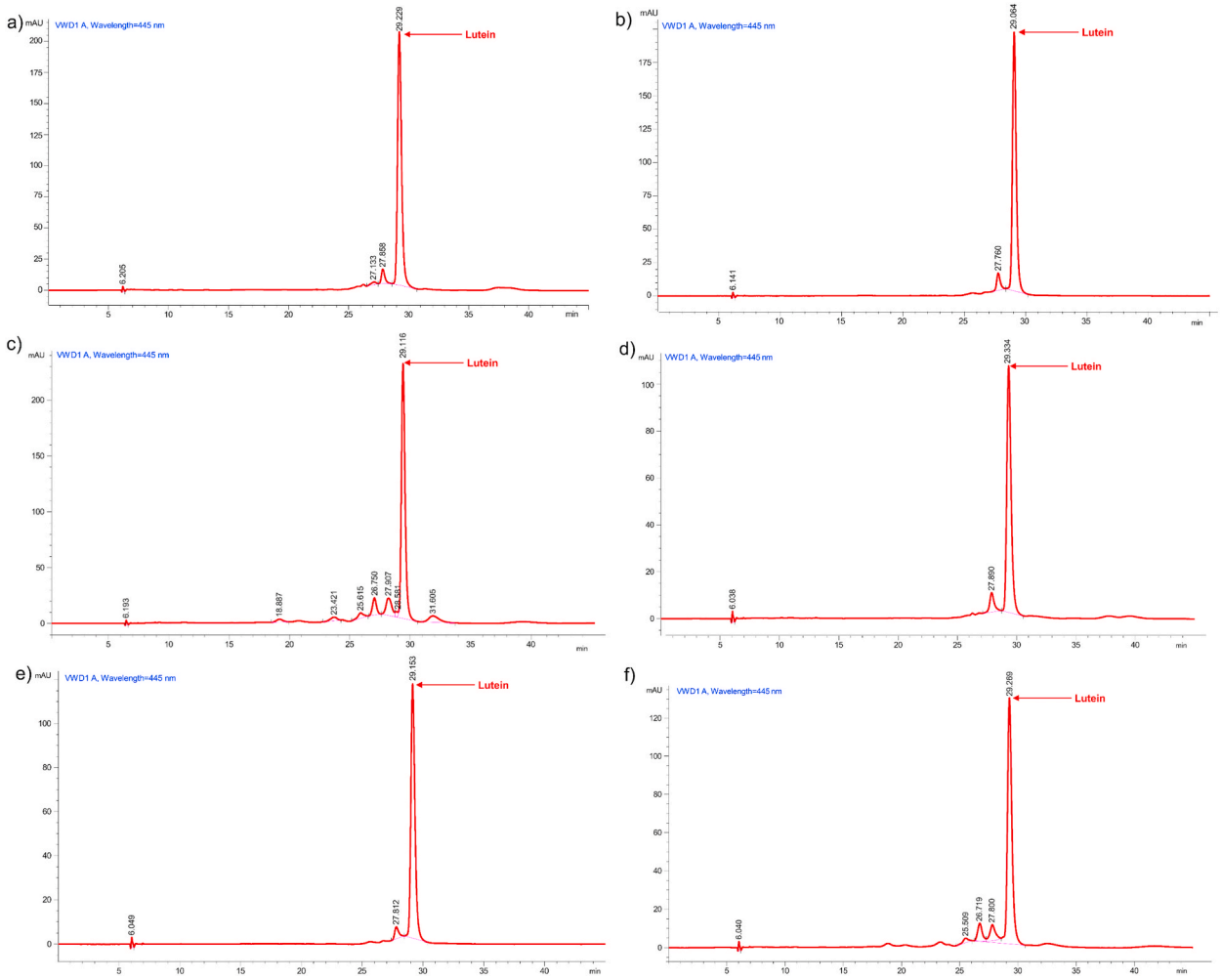

Fig. 2. HPLC chromatograms of lutein standard 20 μg/mL (a), HPLC-purified NN (b), and NN (c) after 0-week storage, and lutein standard 20 μg/mL (d), HPLC-purified NN (e), and NN (f) after 2 weeks storage at –80 °C.

which led to rapid degradation at the first storage stage [42]. At cold storage temperatures, SLs showed lower degradation and thus less prooxidant activity than CL. However, after 7 weeks of storage at 25 °C and 40 °C, SLs and CL were completely degraded, indicating that thermal deterioration is the limiting factor for lutein stability. High temperatures accelerate the rate of the degradation reaction of lutein and carotenoids. Lutein contains all its double bonds in the form of the *trans* isomer. The trans isomers are partially transformed into the less stable *cis* form [13]. Therefore, low temperatures can decrease the degradation rate of lutein. As demonstrated, the retention of CL after 20 weeks of stable storage at −80 °C, −20 °C, and 4 °C was 14.7%, 14.7%, and 7.83%, respectively. Under the same conditions, the retention of SLs at −80 °C, −20 °C, and 4 °C was 39.4–42.0%, 28.3–41.0%, and 2.1–30.7%, respectively. The retention of lutein in SLs is between 3 and 4 times higher than that of CL. These findings show that SLs undergo less oxidation than purified lutein sold commercially, which could be attributed to the different form of lutein binding found in nature and the lower purity level of the SLs extract.

3.3. Degradation kinetic analysis

Knowledge of degradation kinetics is useful for predicting potential changes in food quality during storage. During 0-48-week storage periods, the half-life of lutein samples was used as an indicator to quantify the storage stability of SLs and CL. The rate constant (k) and its activation energy (Ea) were determined to predict the influence of the temperature. According to Table 2, lutein degradation increased with increasing temperature. The rate constant for degradation (k) was highest at 40 °C and lowest at −80 °C. These findings suggest that the lower the storage temperature is, the higher the lutein stability becomes. Both types of lutein are prone to temperature decomposition in the same fashion, especially at 25 °C and 40 °C. The activation energy for the SL breakdown reaction is 4.6–9.5 times higher than CL. When compared to CL, SLs have longer half-lives at low temperatures. As a result, the half-life and activation energy of SLs indicate that they will degrade more slowly than CL (Table 2). However, at storage temperatures of 25 °C and

Table 2
Comparison of rate constant (k), half-life ($t_{\frac{1}{2}}$), and activation energy (Ea) in CL and SLs from 5 varieties of yellow silk cocoons stored at different temperatures.

Sample	Temperature (°C)	Order	k_1	Half-life (week)	Ea_1 (kJ/mol)	k_2	Half-life (week)	Ea_2 (kJ/mol)
Commercial Lutein	−80	2-stage First order	0.6287	1.10	2.266	0.0075	92.38	0.581
	−20		0.6584	1.05		0.0124	55.88	
	4		1.1020	0.63		0.0069	100.43	
	25	Second order	23.897	N/A	N/A	N/A	N/A	N/A
	40		187.01	N/A		N/A	N/A	N/A
Nang Noi	−80	2-stage First order	0.3749	1.85	1.032	0.0035	198.46	10.479
	−20		0.4518	1.53		0.0074	93.72	
	4		0.4484	1.55		0.0366	18.94	
	25	First order	1.2213	0.57	N/A	N/A	N/A	N/A
	40		2.5584	0.27		N/A	N/A	N/A
Nang Tui	−80	2-stage First order	0.3906	1.77	0.572	0.0008	868.63	12.513
	−20		0.4716	1.47		0.0024	289.01	
	4		0.4147	1.67		0.0120	57.77	
	25	Second order	280.06	0.07	N/A	N/A	N/A	N/A
	40	First order	1.5576	0.44		N/A	N/A	N/A
Nang Sew	−80	2-stage First order	0.3295	2.10	1.148	0.0022	315.66	9.569
	−20		0.3872	1.79		0.0041	169.11	
	4		0.4107	1.69		0.0193	35.91	
	25	First order	0.6386	1.09	N/A	N/A	N/A	N/A
	40		2.5638	0.27		N/A	N/A	N/A
Nang Lai	−80	2-stage First order	0.3806	1.82	0.450	0.0026	266.80	4.412
	−20		0.4054	1.71		0.0039	177.77	
	4		0.4150	1.67		0.0067	103.45	
	25	First order	0.9189	0.75	N/A	N/A	N/A	N/A
	40		1.6412	0.42		N/A	N/A	
Samrong	−80	2-stage First order	0.3382	2.05	0.643	0.0029	239.58	11.628
	−20		0.3670	1.89		0.0022	315.26	
	4		0.3842	1.80		0.0655	10.58	
	25	Second order	563.50	N/A	N/A	N/A	N/A	N/A
	40	First order	2.0278	0.34		N/A	N/A	

Note: N/A is not available.

Table 3
Comparison of ABTS, FRAP and DPPH of SLs and CL.

Sample	IC_{50} of ABTS (mM) ± SD	IC_{50} of Fe^{3+}(mM) ± SD	IC_{50} of DPPH (mM) ± SD	Phenolic compounds (mg GAE/g lutein) ± SD
BHA	1.023 ± 0.023^{c}	0.041 ± 0.001^{f}	0.455 ± 0.002^{b}	N/A
Tocopherol	1.147 ± 0.021^{b}	0.110 ± 0.005^{a}	0.464 ± 0.003^{b}	N/A
CL	1.401 ± 0.116^{a}	0.105 ± 0.005^{a}	0.782 ± 0.009^{a}	7.693 ± 1.124^{d}
Nang Noi	0.590 ± 0.020^{d}	0.062 ± 0.003^{cd}	0.228 ± 0.003^{d}	147.669 ± 0.904^{a}
Nang Tui	0.612 ± 0.010^{d}	0.056 ± 0.002^{de}	0.231 ± 0.002^{d}	137.904 ± 2.726^{b}
Nang Sew	0.507 ± 0.006^{d}	0.053 ± 0.002^{e}	0.139 ± 0.002^{f}	139.182 ± 1.531^{b}
Nang Lai	0.627 ± 0.021^{d}	0.068 ± 0.010^{bc}	0.239 ± 0.003^{c}	130.870 ± 1.446^{c}
Samrong	0.636 ± 0.012^{d}	0.073 ± 0.004^{b}	0.164 ± 0.006^{e}	146.346 ± 2.987^{a}

Note: SD: standard deviation, $n = 3$.
[a, b, c, d, e, f] Data are significant difference according to SPSS at $p < 0.05$.

40 °C, when the energy from the surroundings was excessive, the lutein degraded rapidly, as shown in Fig. 1.

According to Ref. [19], lutein in silkworms naturally exists in a protein-binding form, making it more water-soluble and stable than the carotenoid or protein alone. Lutein accounts for 90% of the bound carotenoids in the carotenoid binding protein isolated from the silk glands of *Bombyx mori* larvae [43]. The bound protein is mostly removed during the extraction process, leaving the binding site of such lutein with residue protein peptides potentially active [28]. Furthermore, when analyzed by gas chromatography, the SL extracts were not purified; they contained 10.41% w/w fatty acids, mostly oleic acid, palmitic acid, and linoleic acid, with the remainder being insoluble unidentified wax materials [22,44]. There have been similar reports that the aqueous solubility and chemical stability of purified lutein were improved after interacting with whey protein isolate (WPI) and sodium caseinate (SC) through hydrophobic interaction [45]. During the 16-day storage period, milk proteins, particularly SC, were found to protect lutein from oxidation and breakdown better than WPI. Through the esterification of the O–H group with saturated fatty acids, lutein esters or lutein fatty acid esters are able to retain antioxidant activity and become more stable than the unesterified or free form lutein [16,40,46], suggesting the advantage of using lutein esters over the saponified form [40]. Aside from the protein binding form found in nature, the presence of residue peptides, and the hydrophobic mixture, the SLs extracts also contained a high concentration of phenolic compounds, which could help increase their overall antioxidant activities and stability (Table 3). The phenolic compounds are antioxidants via the

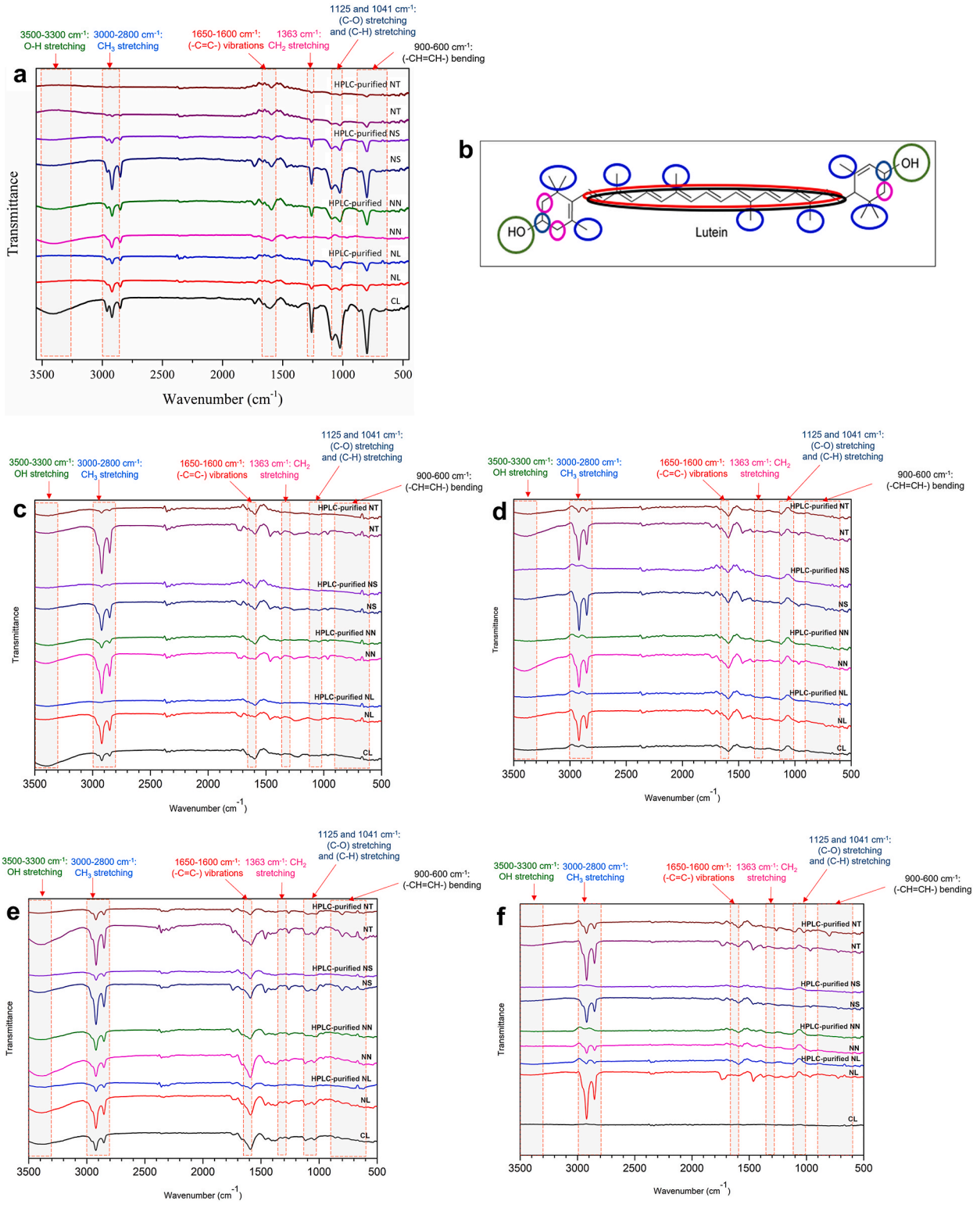

Fig. 3. FTIR spectra of CL, SLs and HPLC-purified SLs from NT, NS, NN, and NL (a) 0 week, (b) Schematic representation of structure of lutein match the functional groups, (c) after stored at −80 °C for 1 week, (d) after stored at −80 °C for 2 weeks, (e) after stored at 25 °C for 1 week, (f) after stored at 25 °C for 2 weeks.

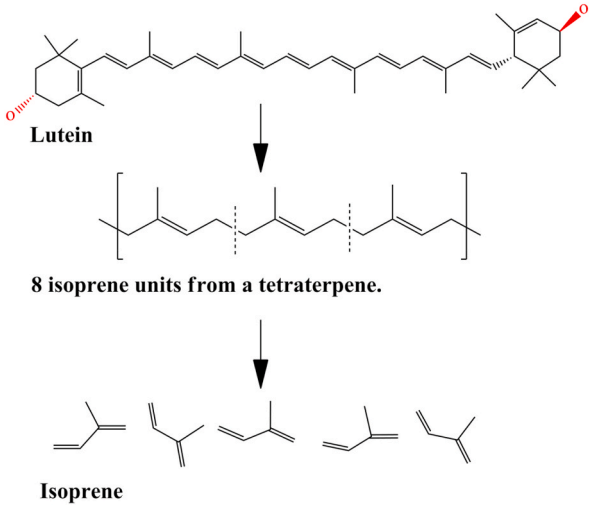

Fig. 4. Schematic representation of lutein degradation at 2 weeks [41].

hydrogen-donating abilities of the phenolic hydroxyl groups [47]. Some phenolic acids improved lutein stability under different conditions (dark/light and heat) via non-covalent interactions (hydrogen bonds and van der Waals forces) [48]. Based on this information, several favorable parameters found in SLs may contribute to their stability and antioxidant activity.

3.4. Molecular structure and chemical bonding of lutein during storage

3.4.1. Fourier transform infrared spectroscopy (FTIR)

An FTIR spectrometer was used in this study to identify functional groups. The effects of storage temperature and time over the first two weeks were topics of interest due to the unique exponential degradation of lutein before reaching a plateau. The stability of CL, SLs, and HPLC-purified SLs from NT, NS, NN, and NL stored at – 80 °C and 25 °C under vacuum was studied. The FTIR spectra of CL, SLs, and HPLC-purified SLs at 0 week are presented in Fig. 3a. All lutein samples displayed distinct peaks at 3500–3300, 3000–2800, and 1650-1600 cm^{-1}, corresponding to the stretching vibrations of the hydroxyl group (O–H stretching), the hydrogen-carbon (C– H stretching), and the conjugated double bond (-C=C- vibration) [49–51]. Peaks at 1363, 1125, and 1041 cm^{-1}, correspond to CH_2, C–O, and C–H stretching, respectively. Furthermore, the band from 900 to 600 cm^{-1} is assigned to –CH=CH-bending [52]. According to Fig. 3b, the structure of lutein corresponds to the functional groups from FTIR spectra. When compared to SLs and HPLC-purified SLs, the stretching vibrations of CH_2, C–O, and, C–H stretching, and –CH=CH- bending of CL gave the highest intensity peaks at 1363, 1125, 1041, and 900 to 600 cm^{-1}, respectively.

The FTIR spectra of all lutein samples showed all characteristic lutein peaks when stored at −80 °C for the first and second weeks. However, the peak intensities at 1363, 1125, 1041, and 900-600 cm^{-1} were reduced for all samples (Fig. 3c and d). According to Fig. 3e, all lutein samples showed all characteristic lutein peaks after being stored at 25 °C for the first week, but peak intensities at 1363, 1125, 1041, and 900-600 cm^{-1} of all samples were reduced. For the second week at 25 °C, CL and HPLC-purified SLs showed similar low intensities of all characteristic peaks, with the disappearance of the vibrational band of O–H stretching, corresponding to lutein degradation at 3500-3300 cm^{-1}, 3000-2800 cm^{-1}, and 1650-1600 cm^{-1}. However, all SLs had a higher peak intensity between 3000 and 2800 cm^{-1}. These findings suggest that SLs may be more resistant to oxidation than CL and HPLC-purified SLs, possibly due to the electron-donating ability of phenolic compounds [39,47]. At the first week of storage at 25 °C, lutein retention in CL was significantly lower than that of all SLs and HPLC-purified SLs ($p < 0.05$), whereas lutein retention in HPLC-purified SLs was significantly lower than that of SLs ($p < 0.05$) (data not shown). The difference in lutein retention between CL and HPLC-purified SLs could be attributed to their naturally occurring structural differences. On the other hand, the difference between SLs and HPLC-purified SLs was due to the mixed compositions in SLs, which could contribute to the delay in lutein degradation. CL remained significantly lower in the second week at 25 °C than NL, NN, and NT, as well as HPLC-purified NN ($p < 0.05$), as storage temperature became a dominant factor. The most common cause of lutein or carotenoids is oxidation, usually occurring via oxidation reactions. In general, the lutein oxidation mechanism is very complicated due to several factors (temperature, light, oxygen, and time). High temperature remains a major challenge throughout the supply chain as other factors can be controlled and managed. Lutein can undergo thermal degradation after being manufactured. It is still a critical phenomenon for lutein.

The oxidative process leads to the formation of isoprene, a compound containing less than 40 carbon atoms [13] Fig. 4. However, the peak of isoprene disappeared at 1650-1600 cm^{-1} (conjugated double bond, –C=C–) in the IR spectrum because alkene was not detected from FTIR measurement. As a result, functional groups, and bioactive properties of lutein were lost [13]. As a result, formulations must be designed to ensure lutein stability during the first stage of degradation to optimize final product storage. HRMS should be used to investigate oxidation products and molecular weight to gain a better understanding of the first stage of lutein

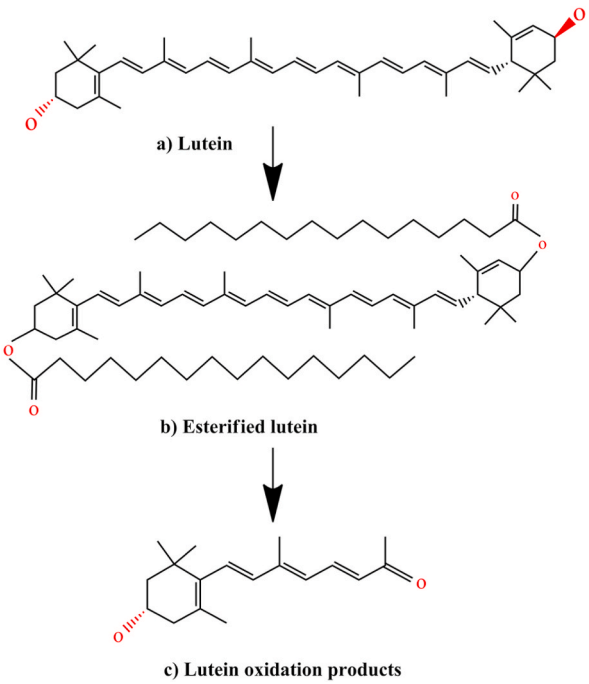

Fig. 5. The structure of all-trans lutein (a), lutein esterified with fatty acids, including palmitic acid (b), and its oxidation products (c) [41].

degradation.

3.4.2. High-resolution mass spectrometry (HRMS)

Peaks of lutein and its fragments were examined in the scanning range of 50–650 m/z. Adjusted detector parameters provide adequate ion signal intensity for peak detection. Under the specified circumstances, a strong peak at 591 m/z for lutein ($M^+ + Na^+$) was identified. The fragmented ions generated from lutein in CL, SLs, and HPLC-purified SLs when exposed to high temperature (25 °C for two weeks) are oxidation products ($C_{18}H_{26}O_2$: 297 m/z) [41]. The FTIR spectra of lutein degradation correspond to the distinct fragmented ions produced only by lutein in CL and HPLC-purified SLs, not all SLs. SLs were lutein mixtures with residue protein peptides, lipids, insoluble wax components, and phenolic compounds [22,28], which made them more resistant to deterioration, particularly during the first stage of degradation [40]. Furthermore [40,46], have shown that esterifying lutein with fatty acids can improve its solubility and potentially stabilize it (Fig. 5). Palmitic acid and stearic acid were the most abundant saturated fatty acids in SLs. These fatty acids would also improve such an important step in lutein bioavailability [22].

Furthermore, Table 3 compares the antioxidation of SLs to CL as a result of this study. In the ABTS, FRAP, and DPPH assays, the 50% inhibitory concentrations (IC_{50}) of all SLs were 2.2–2.8, 1.4–2.0, and 3.3–5.6 times lower than those of CL, respectively. SLs were also found to be significant ($p < 0.05$) when compared to BHA and tocopherol. Furthermore, the phenolic content of SLs was 17.0–19.2 times greater than that of CL. It could be that silkworms can only feed on organic mulberry leaves, which contain a high concentration of phenolic compounds [42,53]. According to the findings of this study, SLs are composed of fat residues, protein peptides, and some phenolic compounds. This combination may help lutein stability by shortening the degradation period during the first stage of storage and slowing the rate of degradation in the second stage.

4. Conclusion and future perspectives

Temperature is a critical factor influencing the storage stability of lutein. High temperature enhances the oxidation reaction that leads to the degradation of lutein. Similar oxidation products were found in both HPLC-purified SLs and CL after two weeks at 25 °C. The rate of lutein degradation followed two-stage first-order reaction kinetics. However, SLs were more stable than CL, which could be attributed to the original lutein structure and chemical bonding: lutein-binding protein vs. lutein ester and the impurity mixture. This study suggests that yellow silk cocoons can be used as a source of lutein in Thailand because they are consumed locally and can be obtained as an extract or in their natural protein-binding form.

Value of the data

- Lutein isolated from its naturally occurring protein-binding form is more resistant to oxidation than the lutein ester form, especially the first stage of degradation.

- After being purified, lutein in its naturally occurring forms had no effect on the types of oxidation-derived compounds.
- Regardless of the forms that lutein naturally occurs in, high temperatures can have a significant impact on its stability and limit its shelf life.
- The source of lutein influences its antioxidant activity, emphasizing the importance of naturally occurring forms. As a result, protein-binding silk lutein has significantly higher antioxidant activity than naturally occurring ester lutein.
- Yellow silk cocoons are another excellent source of lutein for supplements.

Author contribution statement

Waree Manupa, Rachasit Jeencham: Performed the experiments; Analyzed and interpreted the data.

Juthathip Wongthanyakram: Performed the experiments; Wrote the paper.

Manote Sutheerawattananonda: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

The Suranaree University of Technology gave financial assistance and equipment, medications, or supplies to Waree Manupa, Juthathip Wongthanyakram, and Rachasit Jeencham. The Agricultural Research Development Agency gave financial assistance to Waree Manupa and Rachasit Jeencham. The National Research Council of Thailand offered financial support to Manote Sutheerawattananonda. The Agricultural Research development agency has been granted patent number US9018422B2 by Manote Sutheerawattananonda.

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