

Color Loss in Ethanolic Solutions of Chlorophyll *a*

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The present study investigated the stability of green color in 1–60% ethanolic solutions of chlorophyll *a*. Kinetics studies were performed. The results show that the color loss follows first-order reaction kinetics, similar to that in aqueous systems, with a reaction rate constant between 0.013 and 13.575 day⁻¹ at 20 °C and an activation energy between 18.4 and 85.1 kJ mol⁻¹. The rate of color loss increases with the temperature and varies with the ethanol concentration. It reaches the maximum at 40% ethanol concentrations. The difference among the absorption spectra of the ethanolic solutions suggests that the interaction between water and ethanol molecules may be the major factor affecting the color stability of chlorophyll in the solutions. The bathochromic shift is most obvious around 40% ethanol concentration. We postulate that the formation of clusters of water–ethanol molecules is responsible for the increase in the rate of color loss.

KEYWORDS: Ethanol; chlorophyll; color loss; kinetics; bathochromic shift

INTRODUCTION

Color is responsible for visual recognition. It is also a major quality attribute of food. Chlorophyll is one of the most widely occurring natural pigments. Its green color is often taken as a quality indicator of green fruits, vegetables, and their processed products. Chlorophyll degrades and discolors easily during food processing. Foods containing chlorophyll are difficult to be processed with good retention of the characteristic greenness. Thermally processed green vegetables usually exhibit poor color quality as compared to the fresh ones. The color changes from bright green to olive brown during processing are due to the degradation of chlorophyll to pheophytin by the replacement of magnesium in the chlorophyll by hydrogen and the further formation of the degradation products, such as pheophorbides and chlorins (*I*). The stability and degradation of chlorophyll in various foods has been a subject of interest for scientific studies. Most of the studies have been performed in raw materials or aqueous systems. For example, the major factors in the processing of vegetable leaves, purees, and juices that affect the stability of chlorophyll were found to be temperature, pH, and water activity, and the degradation of chlorophyll in aqueous solutions was found to follow the first-order reaction kinetics (2–8).

Some alcoholic beverages, such as Chinese bamboo-leaf-green liqueur, New Zealand kiwifruit liqueur, and a few wine coolers, are made by soaking or mixing a chlorophyll-containing raw material or semi-product in potable alcohol. Being a solvent in food products, ethanol is second to water only as far as importance is concerned. However, no literature regarding to the stability of chlorophyll in ethanolic systems have been reported. Therefore, the present study was aimed to investigate the stability of chlorophyll in ethanolic solutions.

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MATERIALS AND METHODS

Preparation and Storage of the Ethanolic Solutions of Chlorophyll *a*. Ethanolic solutions at 1, 10, 20, 30, 40, 50, and 60% ethanol concentrations containing 0–6 mg mL⁻¹ chlorophyll *a* (Wako Pure Chemicals Industries, Ltd., Osaka, Japan) were prepared to evaluate the relationship between chlorophyll *a* content and the color of the solutions.

Ethanolic solutions at 1, 10, 20, 30, 40, 50, and 60% ethanol concentrations containing 4 μg mL⁻¹ chlorophyll *a* were also prepared. Each 4.0 mL aliquot of the solution was filled into a 4.5 mL brown glass vial. The vial was sealed with a cap with a Teflon gasket. The color of the ethanolic solutions in storage at 20, 30, 40, or 50 °C up to 60 days was measured periodically.

Color Measurement. The color was determined using a color and color difference meter (model ZE2000, Nippon Denshoku, Tokyo, Japan). A penetrable whiteboard (*x*, 93.18; *y*, 95.18; *z*, 112.18) was used as the standard. Hunter's *-a* value was read to signify the degree of greenness.

Kinetic Calculations. The changes in the Hunter *-a* value in the solutions during storage were fitted into the following first-order kinetics equation:

$$\ln(-a/-a_0) = -kt \quad (1)$$

Table 1. Correlation between Hunter's *-a* Value and Chlorophyll *a* in Ethanolic Solutions

ethanol concentration (% v/v)	standard curve equation ^a	R ²
1	$y = -2.2871x - 0.3404$	0.9967
10	$y = -2.2892x - 0.3592$	0.9968
20	$y = -2.4484x - 0.4668$	0.9946
30	$y = -2.5777x - 0.5299$	0.9935
40	$y = -2.6634x - 0.5251$	0.9946
50	$y = -2.5621x - 0.1437$	0.9997
60	$y = -2.7041x - 0.5048$	0.9965

^a *y* is Hunter's *-a* value, and *x* is the concentration of chlorophyll *a*.

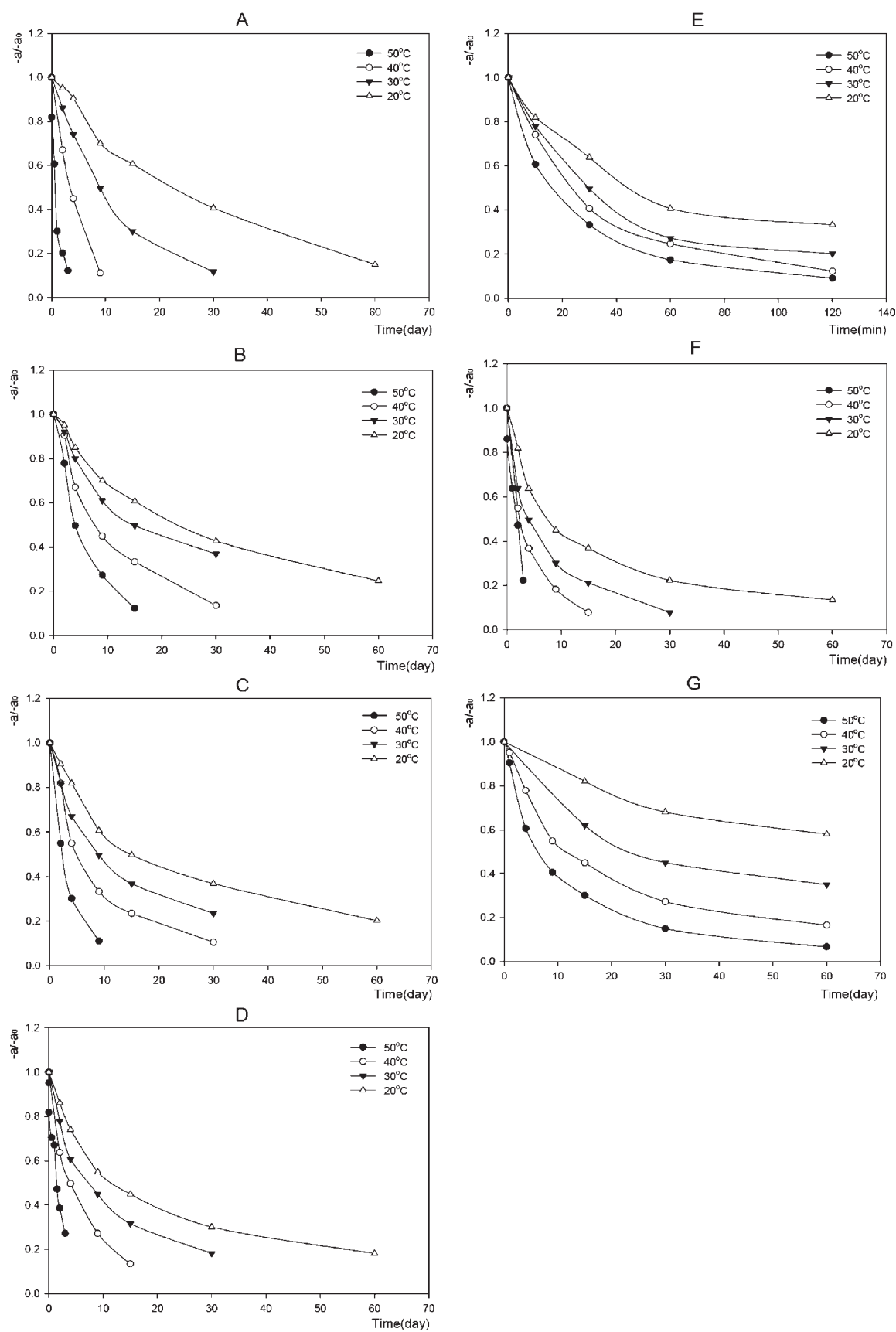


Figure 1. Color retention in (A) 1%, (B) 10%, (C) 20%, (D) 30%, (E) 40%, (F) 50%, and (G) 60% ethanolic solutions of chlorophyll a during storage at 20, 30, 40, and 50 °C.

for the evaluation of k , where $-a$ and $-a_0$ are Hunter's $-a$ values at times t (dimensionless) and 0, respectively. $-a/-a_0$ expresses the percent

loss of greenness, and k is the rate constant for the loss of green color (day^{-1}).

The dependence of the color loss upon temperature was determined by the Arrhenius equation

$$k = k_0 e^{-E_a/RT} \quad (2)$$

where E_a is the activation energy of the reaction (kJ mol^{-1}), R is the universal gas constant ($8.3145 \text{ J mol}^{-1} \text{ K}^{-1}$), T is the absolute temperature (K), and k_0 is the pre-exponential factor.

Spectrum Analysis. The spectrum analysis of the model solutions was conducted using a spectrophotometer (Model Helios Alpha, Spectronic Unicam, Cambridge, U.K.) to scan over the wavelength range between 300 and 900 nm.

Statistical Analysis. Each experiment was performed in triplicate. The results were expressed as mean \pm standard error and analyzed by one-way analysis of variation (ANOVA) and Duncan's new multiple range tests.

RESULTS

Reaction Order. There is significant correlation ($R^2 > 0.99$) between the chlorophyll *a* content and visual color parameter $-a$ at each ethanol concentration (Table 1), indicating that Hunter's $-a/a_0$ value is a good measure of chlorophyll degradation and color loss. Nisha et al. (1) reported the first-order kinetics for the loss of greenness in spinach puree containing no ethanol. Figure 1 shows color retention in the various ethanolic solutions of chlorophyll *a* storage at 20, 30, 40, and 50 °C. The semi-logarithmic plotting (data not shown) of the loss of green color (Hunter's $-a/a_0$) versus time in the present study revealed that the loss of greenness in the ethanolic solutions of chlorophyll *a* follows the first-order kinetics.

Rate Constant. Table 2 shows the rate constants of color loss in the ethanolic solutions of chlorophyll *a* at various temperatures. The rate of color loss increases with the increase in the temperature. For example, the rate constants in 1, 10, 20, 30, 40, 50, and 60% ethanolic solutions at 20 °C storage are 0.031, 0.024, 0.027, 0.028, 13.575, 0.032, and 0.013 day^{-1} , respectively. They are increased to 0.782, 0.145, 0.229, 0.553, 33.179, 0.710, and 0.061 day^{-1} , respectively, when the storage temperature is elevated to 50 °C.

Chlorophyll is insoluble in pure water (9). In the present study, 1% ethanol solution containing 4 μg of chlorophyll *a* mL^{-1} was prepared in lieu of the aqueous solution as a reference. The rate constants of color loss in 10–30% ethanolic model solutions are lower than that in the 1% solution at the same temperature (Table 2). The rate of color loss jumps to the maximum value around 40% ethanol concentration. Among model solutions with ethanol concentrations between 10 and 40%, a higher ethanol concentration corresponds to a higher rate of color loss. A big drop in the rate of color loss occurs when the ethanol concentration changes from 40 to 50%. A much smaller decrease in the rate of color loss occurs when the ethanol concentration further increases to 60%.

Activation Energy. The slope of the semi-logarithmic plot of the rate constant versus the inverse of time is the activation energy for color loss (Figure 2). The results show that chlorophyll *a* has the highest activation energy at 85.10 kJ mol^{-1} in 1% ethanolic solution and the lowest value at 18.43 kJ mol^{-1} in 40% ethanolic solution.

Spectral Analysis. Figure 3 shows the visible spectra of chlorophyll *a* solutions with different ethanol concentrations. Each spectrum is characterized by two major absorption peaks. There are some differences in the maximum absorption wavelength among different spectra. The spectra of the solutions with ethanol concentrations ranging from 1 to 30% are relatively close to each other. However, when the ethanol concentration increases to 40%, noticeable bathochromic shifts of the absorption peaks from 400 to 432 nm and from 650 to 670 nm occur. When the ethanol concentration further increases to 50%, the bathochromic

Table 2. Rate Constants and Activation Energies for Color Loss in Ethanolic Solutions of Chlorophyll *a*

ethanol concentration (%, v/v)	temperature (°C)	rate constant (day^{-1})	activation energy (E_a , kJ mol^{-1})
1	20	0.031 \pm 0.002	85.1
	30	0.053 \pm 0.003	
	40	0.231 \pm 0.021	
	50	0.782 \pm 0.113	
10	20	0.024 \pm 0.001	46.2
	30	0.033 \pm 0.003	
	40	0.064 \pm 0.105	
	50	0.145 \pm 0.012	
20	20	0.027 \pm 0.002	53.6
	30	0.046 \pm 0.010	
	40	0.074 \pm 0.007	
	50	0.229 \pm 0.033	
30	20	0.028 \pm 0.003	75.5
	30	0.054 \pm 0.003	
	40	0.124 \pm 0.027	
	50	0.553 \pm 0.020	
40	20	13.575 \pm 1.760	18.4
	30	19.482 \pm 0.223	
	40	23.739 \pm 0.959	
	50	33.179 \pm 17.60	
50	20	0.032 \pm 0.002	78.2
	30	0.077 \pm 0.002	
	40	0.153 \pm 0.027	
	50	0.710 \pm 0.073	
60	20	0.013 \pm 0.001	44.2
	30	0.018 \pm 0.002	
	40	0.030 \pm 0.002	
	50	0.061 \pm 0.009	

mic shifts disappear and the absorption peaks move back. The spectrum of model solutions varies with the ethanol concentration, suggesting that ethanol may interact with water or chlorophyll in the solution in addition to being a solvent.

DISCUSSION

Chlorophyll retention in green vegetables serves as a good index for evaluating the severity of thermal processing (10). There is good correlation between the degradation of chlorophyll and the loss of green color (11, 12). Many researchers have evaluated the kinetics of chlorophyll degradation and color loss in vegetables, such as broccoli (13), spinach (1), and green peas (8). They all concluded that chlorophyll degradation in aqueous solutions follows the first-order kinetics. The results of the present study show a similar behavior of chlorophyll *a* in ethanolic solutions (Figure 1).

The results in rate constant determination indicate that the temperature is an important factor affecting the stability of chlorophyll in ethanolic solutions and that low temperature favors its retention (Table 2), similar to previous reports in aqueous systems (12, 14–16).

Previous studies have reported wide variation in the activation energies for color loss of green vegetables. Activation energies of 28.6, 41.2, and 34.0 kJ mol^{-1} for spinach puree, mustard leaves, and a mixed puree, respectively, were reported by Ahmed et al. (7). Activation energies for color loss in broccoli juice (13) and green chili puree (17) were reported to be 72.0 and 11.3–16.0 kJ mol^{-1} , respectively. Activation energies varied from 34.0 to 49.8 kJ mol^{-1} with pH (5.5–7.5) in the blanching of green peas (8). The results in the present study, from 18.4 to 85.1 kJ mol^{-1} , are of similar magnitude.

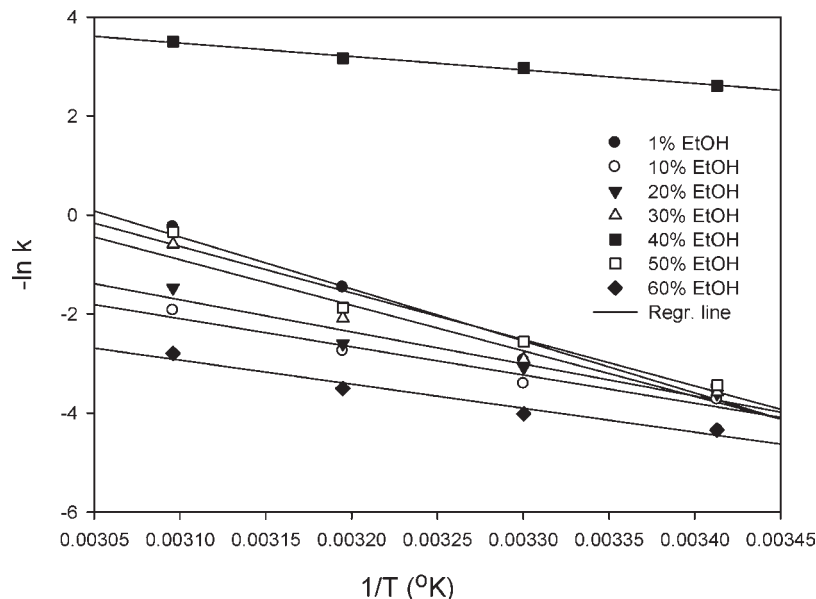


Figure 2. Arrhenius plot for color loss in ethanolic solutions of chlorophyll *a*.

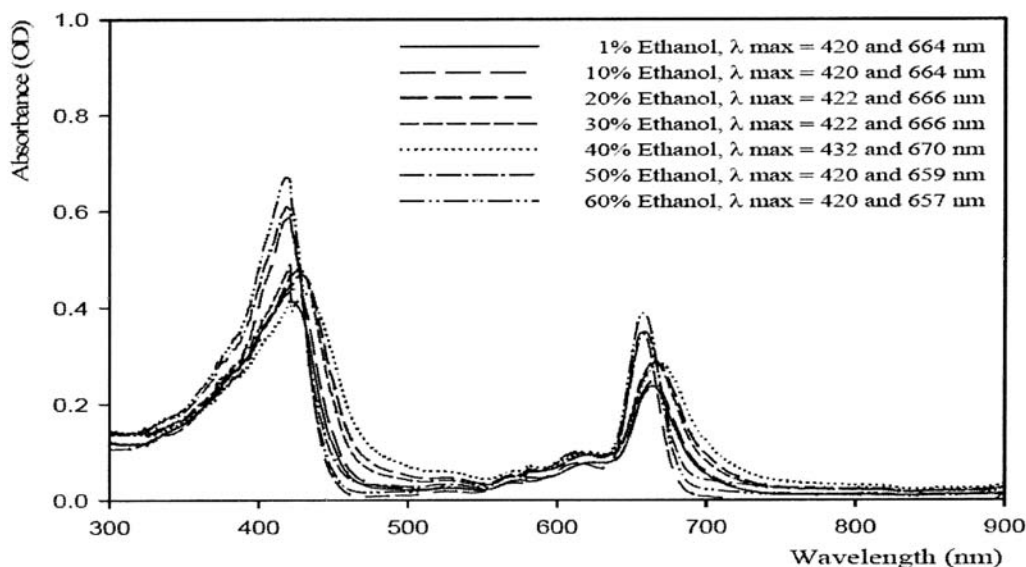


Figure 3. Ultraviolet–visible spectra in ethanolic solutions of chlorophyll *a*.

We postulate that solutions with different ethanol/water ratios compose different chemical environments and influence the stability of chlorophyll. We also postulate that the interaction between ethanol and water molecules is strongest around 40% ethanol concentration because the highest rate of color loss and the largest shift in spectrum occurs at or around this concentration. This postulation can be supported by the following literature. Frank and Ives (18) found that the 40% ethanol solution is more viscous than the ethanol solutions at other concentrations. They proposed that the number of hydrogen bonds between ethanol and water molecules reaches the highest at 40% ethanol concentration. Koga and Yoshizumi (19) used differential scanning calorimetry to investigate the characteristics of ethanol/water mixtures. The highest heat absorption peak appeared in the thermal chromatograph profile of the 40:60 (v/v) ethanol/water mixture. When the ethanol concentration further increased, the heat absorption decreased gradually, reached the lowest value at 60% ethanol concentration, and increased again afterward. The interaction between ethanol and water molecules, with the

appearance of the strongest interaction at 40% ethanol concentration, was then proposed to explain the phenomenon.

In the ethanol–water mixture, each ethanol molecule contains a hydrophilic hydroxyl group, which is able to form hydrogen bonding with water molecules, and an alkyl chain, which confers a certain degree of hydrophobicity upon the molecule (20). Erdey-Gruz and Majthenyi (21) proposed the existence of $\text{EtOH}-n\text{H}_2\text{O}$ ($n = 4$ or 8). Takahashi and Watanabe (22) investigated the interaction of chitin with chlorophyll *a* in ethanol–water mixtures and reported the bathochromic shift of the chlorophyll *a* spectrum in response to the ethanol concentration, including the occurrence of the largest shift at 44% ethanol concentration. They attributed the bathochromic shift to the formation of clusters of ethanol and water molecules, such as the ethanol hydrate of $[\text{EtOH}-4\text{H}_2\text{O}]$ or $[\text{EtOH}-8\text{H}_2\text{O}-\text{EtOH}]$. The present study noticed the concomitant occurrence of the most pronounced color loss and bathochromic shift in the 40% ethanolic solution among all of the solutions tested that do not include 44% ethanol concentration (Figure 3). We postulate that the formation of

clusters of solvent molecules may also decrease the stability of chlorophyll molecules, as shown by the increased rate in color loss, and that temperature is an important environmental factor to alter the association between ethanol and water molecules. The increase in storage temperature may reduce the association between ethanol and water molecules and promote the color loss in ethanolic solutions of chlorophyll as a consequence (**Table 2**).

In conclusion, the loss of color and the degradation of chlorophyll *a* in ethanolic solution follows the first-order kinetic. Chlorophyll *a* exhibits the fastest color loss around 40% ethanol concentration. We suggest avoiding the soaking of chlorophyll-containing materials in potable alcohol around 40% ethanol concentration in the making of liqueur. Further investigation in the mechanism for ethanol concentration to influence chlorophyll stability will be worthwhile.

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