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Effects, Quenching Mechanisms, and Kinetics of Water Soluble Compounds in Riboflavin Photosensitized Oxidation of Milk

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To protect the nutrient and flavor stability of milk under light, the effects of 0, 0.01, 0.03, and 0.05 M 1,4-diazabicyclo[2,2,2]octane (DABCO) and 2,5-dimethylfuran (DMF) on the riboflavin photosensitized oxidation of milk were studied. The oxidation of milk was studied by measuring the headspace oxygen in sample bottles after 3 h of light exposure at 3000 lux. As the concentration of DABCO and DMF, which are water soluble compounds, increased in the sample from 0, 0.01, and 0.03 to 0.05 M, the depleted headspace oxygen content significantly decreased ($P < 0.05$). Steady state kinetic studies of singlet oxygen oxidation showed that the antioxidant activity of DABCO and DMF was due to singlet oxygen quenching. The reaction rate constant of singlet oxygen with milk fat was 8.1×10^5 M^{-1} s^{-1}. Total singlet oxygen quenching rates of DABCO and DMF were 1.5×10^7 and 2.6×10^7 M^{-1} s^{-1}, respectively. DABCO and DMF could be used to slow the reaction between singlet oxygen and milk components to protect nutrients, especially riboflavin, and to improve the oxidative stability of milk fat during storage or processing under light.

KEYWORDS: Quenching mechanism; kinetics; riboflavin; photosensitized oxidation; milk

INTRODUCTION

Milk contains 1.75 mg riboflavin per liter and is the most important source of riboflavin in the American diet (*1*). Milk contributes 40−50% of the total dietary riboflavin in the United States (*2*). Although riboflavin is heat stable, it is very sensitive to light. Milk lost 30% riboflavin to sunlight for 30 min (*3*). The 50% riboflavin of macaroni was lost after 1 day of storage under light. The light intensity was the rate-determining factor for the riboflavin loss in the macaroni (*4*). The wavelength of 450 nm was the most destructive to riboflavin. Riboflavin in milk in a clear bottle or white sachet was lost faster than riboflavin in milk packed in a brown bottle or carton (*5*).

Fluorescent light is commonly used in supermarkets to display dairy products and provides good conditions for light absorption by riboflavin (*6*−*9*). Riboflavin under light can generate reactive oxygen species such as superoxide anions, hydroxyl radical, and singlet oxygen (*10*−*13*). The distribution of the reactive oxygen species formed in a particular riboflavin photosensitized system depends on the availability of oxygen, the concentration of riboflavin, and the presence of other oxidizable reactants or quenchers (*11*−*14*). Reactive oxygen species can cause not only nutrient destruction but also off-flavor formation in foods.

Bradley and Min (*15*) reported the formation of single oxygen by riboflavin using electron spin spectrometry. King and Min (*16*) and Huang and others (*17*) reported the singlet oxygen formation rate by 15 ppm riboflavin in 12% water/88% acetone solution and 40 μM riboflavin in a milk fat aqueous emulsion system. Singlet oxygen directly reacts with electron-rich components such as double bonds of fatty acids to produce undesirable flavor (*15*). Undesirable rancid flavor compounds such as hexanal and 2-heptenal were formed from linoleic acid in soymilk by singlet oxygen oxidation (*17*). The reaction rates between triplet oxygen and singlet oxygen with linoleic acid are 8.9×10^1 and 1.3×10^5 M^{-1} s^{-1}, respectively (*18*). The rate of singlet oxygen with linoleic acid is about 1500 greater than that of ordinary atmospheric triplet oxygen. The rate that soybean oil reacts with singlet oxygen is 1.4×10^5 M^{-1} s^{-1} (*19*).

Milk exposed to natural or artificial light leads to the development of an off-flavor called sunlight flavor (*20*, *21*). Jung and others (*22*) reported that the undesirable sunlight flavor of milk was due to the reaction between the singlet oxygen and the methionine for dimethyl disulfide. The singlet oxygen was formed from the atmospheric triplet oxygen by riboflavin photosensitization.

King and Min (*23*) reported that vitamin D_2 was destroyed by singlet oxygen formed by riboflavin under light and produced a 5,6-epoxide of vitamin D_2. The rapid destruction of riboflavin in foods under light was partly due to the reaction between singlet oxygen and riboflavin (*24*).

The reaction rate between riboflavin and singlet oxygen is 1.01×10^{10} M^{-1} s^{-1} (*18*). This reaction rate is one of the fastest chemical reaction rates. This exceptionally high chemical reaction rate clearly explains why the riboflavin is so easily destroyed under light. The electrophilic singlet oxygen reacts

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with riboflavin to destroy the riboflavin, which has many double bonds in the molecule. The riboflavin photosensitized oxidation of milk destroys vitamin D and riboflavin; produces hexanal, which is responsible for rancid off-flavor; and forms dimethyl disulfide, which is responsible for sunlight off-flavor, within hours under light (*22*, *23*).

The effects of lipid soluble quenchers such as α-, γ-, and δ-tocopherols (*25*), β-carotene, canthaxantine, β-apo-8′carotenal (*26*), lutein, zeaxantine, lycopene, isozeaxantinse, and astaxantine (*19*) on minimizing the singlet oxygen oxidation of vegetable oils have been extensively studied. The effects of water soluble compounds to minimize the singlet oxygen oxidation in foods have been studied in a very limited way. The effects, quenching mechanisms, and kinetics of water soluble quenchers in milk have not been reported. The objective of this research was to determine the effects, quenching mechanisms, and kinetics of the water soluble 1,4 diazabicyclo-[2,2,2]octane (DABCO) and 2,5-dimethylfuran (DMF) on the riboflavin photosensitized oxidation of milk to protect nutrients and flavor quality of milk under light.

MATERIALS AND METHODS

Materials. Milk and cream from a local supermarket, DABCO from Sigma Chemical Co. (St. Louis, MO), and DMF from Aldrich Chemical (Milwaukee, WI) were used.

Sample Preparation for the Effects, Quenching Mechanisms, and Kinetics of DABCO and DMF on Riboflavin Photosensitized Oxidation on Milk. To study the effects of the water soluble compounds DABCO and DMF on the riboflavin photosensitized oxidation of milk, samples of 0, 0.01, 0.03, and 0.05 M DABCO and DMF in milk containing 40 μM riboflavin were prepared in duplicate (*27*).

Thirty milliliters of milk containing DABCO or DMF was transferred into a 35 mL serum bottle. The bottles were sealed airtight with Teflon-coated rubber septa and aluminum seals and exposed to light and dark storage conditions for 3 h. Samples were placed in a light chamber consisting of a rectangular glass (60 cm × 30 cm × 50 cm) liner. Four fluorescent F401F lamps (Sylvania Co., Danver, MA) were placed below the glass chamber where the light intensity at the sample level was 3000 lux (*27*). The temperature of the light chamber was 22 °C. Samples were rearranged in the light chamber every hour to provide uniform light exposure.

The quenching mechanisms and kinetics of DABCO and DMF in riboflavin-photosensitized oxidation of milk were studied by the steady state kinetic equation (*28*). Samples containing 0.10, 0.14, 0.21, and 0.41 M milk fat with 40 μM added riboflavin and 0, 0.01, 0.03, and 0.05 M DABCO and DMF were prepared in duplicate. One mole of milk fat was calculated to be 803 g in 1 L (*29*). Cream containing 36% milk fat was diluted with deionized water to achieve 0.10, 0.14, 0.21, and 0.41 M milk fat. The 30 mL of sample was transferred into a 35 mL serum bottle, sealed airtight, and placed in the light chamber for 3 h. The quenching mechanisms and kinetics of DABCO and DMF in milk were studied by measuring the depleted headspace oxygen of the sample bottle by gas chromatography (*30*). The depleted headspace oxygen content was then expressed as μmol of oxygen per mL of headspace gas. A steady state kinetic equation was applied to determine the quenching mechanisms and kinetics of DABCO and DMF (*31*).

Determination of Headspace Oxygen Depletion. The oxidative stability of milk was determined by measuring the oxygen disappearance in the headspace of the sample bottles (*32*). One milliliter of headspace of the sample was injected into a Hewlett-Packard 5880A gas chromatography equipped with a thermal conductivity detector using a 1 mL gastight syringe (Hamilton Co., Reno, NV). A stainless steel column (1.83 m × 0.32 cm i.d.) packed with 80/100 mesh molecular sieve 13× (Alltech Associates, Inc., Deerfield, IL) was used with helium gas at a flow rate of 30 mL/min. The temperatures of the oven, injector port, and detector were 40, 120, and 180 °C, respectively. The gas chromatographic peak area of oxygen in 1 mL of headspace gas was measured using a Hewlett-Packard 3390 electronic integrator and expressed as electronic counts. One milliliter of air contained 0.20946 mL of oxygen (*33*), and 22400 mL of oxygen equaled 10^6 μmol according to Avogadro's Law. The 0.20946 mL amount of oxygen equaled 9.35 μmol. That is, 1 mL of air contained 9.35 μmol of oxygen. The gas chromatographic peak area of 9.35 μmol of oxygen was measured in electronic counts by injecting 1 mL of air into the gas chromatograph, and electronic counts of 1 μmol of oxygen were calculated. The electronic counts of O_2/mL of headspace gas were then converted to μmol of oxygen.

Statistical Analyses. Depleted headspace oxygen values reported are mean values of duplicate samples. Tukey's range test (SAS, *34*) was used to determine the effects of different levels of DABCO and DMF on the depleted headspace oxygen of milk samples during storage.

Table 1. Effects of 0.01, 0.03, and 0.05 M DABCO and DMF on the Depleted Headspace Oxygen[a] of Milk with 0.10, 0.14, 0.21, and 0.41 M Milk Fat Containing 40 μM Riboflavin after 3 h of Storage under Light at Room Temperature

		depleted headspace oxygen (μmol of O_2/mL of headspace)			
		milk fat concentration (M)			
		0.10	0.14	0.21	0.41
DABCO (M)	0.00	1.79 a	2.32 a	2.90 a	4.10 a
	0.01	0.95 b	1.20 b	1.71 b	2.64 b
	0.03	0.70 c	0.94 c	1.26 c	2.18 c
	0.05	0.52 d	0.64 d	0.97 d	1.73 d
DMF (M)	0.00	1.79 a	2.32 a	2.90 a	4.10 a
	0.01	0.77 b	1.02 b	1.33 b	2.33 b
	0.03	0.47 c	0.62 c	0.91 c	1.58 c
	0.05	0.34 d	0.44 d	0.67 d	1.18 d

[a] Headspace oxygen is the mean value of analyses of duplicate samples; means in a column of the same quencher with different letters are significantly different at $P < 0.05$.

RESULTS AND DISCUSSION

Effects of DABCO and DMF on Riboflavin Photosensitized Oxidation of Milk. The effects of 0, 0.01, 0.03, and 0.05 M DABCO and DMF on the headspace oxygen depletion of milk samples containing 40 μM riboflavin after 3 h of light storage are shown in **Table 1**. The coefficient of variance ($n = 5$) of headspace oxygen determined by gas chromatography was 1.5%. The depleted or reacted headspace oxygen of milk sample with 0.41 M milk fat containing 40 μM added riboflavin stored after 3 h of illumination without quencher was 4.10 μmol. The depleted 4.10 μmol of oxygen is the same as 9.17% oxygen in the air. The 20.946% oxygen in 1 mL of air is equal to 9.35 μmol. However, the oxygen in the headspace air of the same milk stored in the dark for 3 h did not react with milk and was still 20.9%. The 9.17 and 0.0% oxygen reacted with milk during the storage for 3 h under light and in dark, respectively. The oxygen reacted with milk during the storage under light must not be the same oxygen, which did not work with the milk in the dark for 3 h. The riboflavin free milk was prepared by passing milk through the liquid chromatography using Fluorosil as the stationary phase. The headspace oxygen of riboflavin free milk did not react with milk under light or in dark for 3 h. The fat content in the riboflavin free sample was the same as that in the sample with riboflavin. The depleted headspace oxygen of milk containing riboflavin under light was the singlet oxygen formed by the interaction of riboflavin under light and atmospheric triplet oxygen as shown in **Figure 1** (*35*). The singlet oxygen reacted with milk components and depleted during the storage under light. As the concentration of DABCO and DMF increased from 0.0, 0.01, and 0.03 to 0.05 M, the depleted

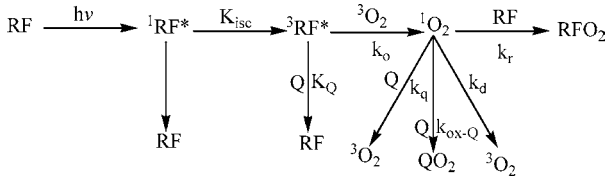

Figure 1. Formation of singlet oxygen from atmospheric triplet oxygen by riboflavin photosensitization. RF, riboflavin; ^{1}RF*, excited singlet state riboflavin; ^{3}RF*, excited triplet state riboflavin; K_{isc}, intersystem crossing rate from singlet state to triplet state; Q, quencher; K_Q, quenching rate; $^3O_2$, triplet oxygen; $^1O_2$, singlet oxygen; k_o, reaction rate between excited triplet state riboflavin and triplet oxygen; k_q, physical quenching rate; k_{ox-Q}, chemical quenching rate; k_d, decaying rate; and k_r, reaction rate between singlet oxygen and riboflavin.

headspace oxygen content significantly decreased ($P < 0.05$) as shown in **Table 1**. DABCO and DMF did not have any effect on the depleted headspace oxygen in milk for the 3 h of storage in dark. The concentrations of 0.01, 0.03, and 0.05 M DABCO or DMF acted as antioxidants in milk containing riboflavin under light. As the fat content increased from 0.10, 0.14, and 0.21 to 0.41 M in the sample, the depleted headspace oxygen increased in all of the samples containing different levels of DABCO and DMF (**Table 1**).

Figure 1 shows the formation of singlet oxygen by riboflavin under light, the reaction of singlet oxygen with food components, and quenching mechanisms of quenchers such as DABCO (*35*). The ground singlet state riboflavin receives the energy from light and becomes the excited singlet state riboflavin. The excited singlet state riboflavin becomes the excited triplet state riboflavin by an intersystem crossing mechanism. The excited triplet state riboflavin reacts with atmosphere triplet oxygen to produce a superoxide anion or singlet oxygen (*13*). Singlet oxygen is electrophilic and reacts with the compounds containing double bonds such as riboflavin and linoleic acid.

Quenching Mechanisms and Kinetics of DABCO and DMF in Riboflavin Photosensitized Oxidation of Milk. The effects of 0, 0.01, 0.03, and 0.05 M DABCO and DMF on the headspace oxygen depletion of 0.10, 0.14, 0.21, and 0.41 M milk fat containing 40 μM riboflavin after 3 h of light storage are shown in **Figures 2** and **3**, respectively. The effects of 0, 0.01, 0.03, and 0.05 M DABCO and DMF on the headspace oxygen depletion of 0.10, 0.14, 0.21, and 0.41 M milk fat containing 40 μM riboflavin after 3 h of storage in dark were not observed (data not shown). As the concentration of DABCO and DMF increased, the depleted headspace oxygen content of the sample bottles decreased during storage under light. If DABCO and DMF reduced riboflavin-photosensitized oxidation of milk by singlet oxygen quenching, the following steady state kinetic equation is established (*35*)

$$\left\{-\frac{d[O_2]}{dt}\right\}^{-1} = \left\{\frac{d[AO_2]}{dt}\right\}^{-1} = K^{-1}\left\{1 + \frac{(k_q + k_{ox-Q})[Q] + k_d}{k_r} \times [A]^{-1}\right\}$$

where K denotes the rate of singlet oxygen formation; AO_2 is oxidized milk fat; k_r is the reaction rate constant of milk fat with singlet oxygen; A is milk fat; k_q is the reaction rate constant of physical singlet oxygen quenching by DABCO; k_{ox-Q} is the reaction rate constant of chemical singlet oxygen quenching by DABCO; Q is the quencher such as DABCO; and k_d is the decay rate of singlet oxygen in water. The plot of $\{-d[O_2]/dt\}^{-1}$ against $[A]^{-1}$ at various [Q] gives a constant y-intercept equal to K^{-1}. Constant intercepts of the plots $\{-d[O_2]/dt\}^{-1}$ against $[A]^{-1}$ at various [Q] are diagnostic of singlet oxygen quenching (*35*). In the presence of quencher, the slope of this plot S_Q is $K^{-1} \times \{(k_q + k_{ox-Q})[Q] + k_d\}/k_r$. Without quencher, the slope (S_O) is $K^{-1}\, k_d/k_r$. The intercepts of the plots are independent of the concentration of quencher, and the slopes are dependent on the concentration of quencher if quenchers quenched singlet oxygen only (*34*). The k_d values are known for many solvents (*36*). Because S_Q/S_O is $\{[(k_q + k_{ox-Q})[Q] + k_d]/k_r\}$, the plot of

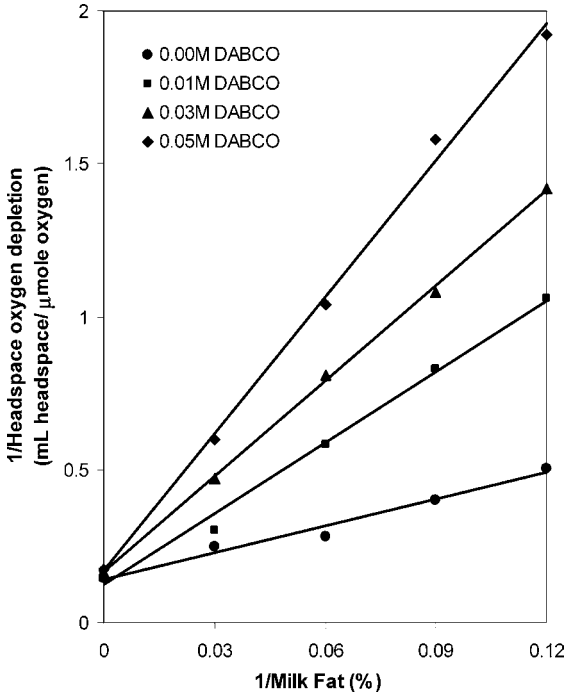

Figure 2. Effect of DABCO on the headspace oxygen depletion of milk with milk fat containing 40 μM riboflavin after 3 h of storage under light at room temperature.

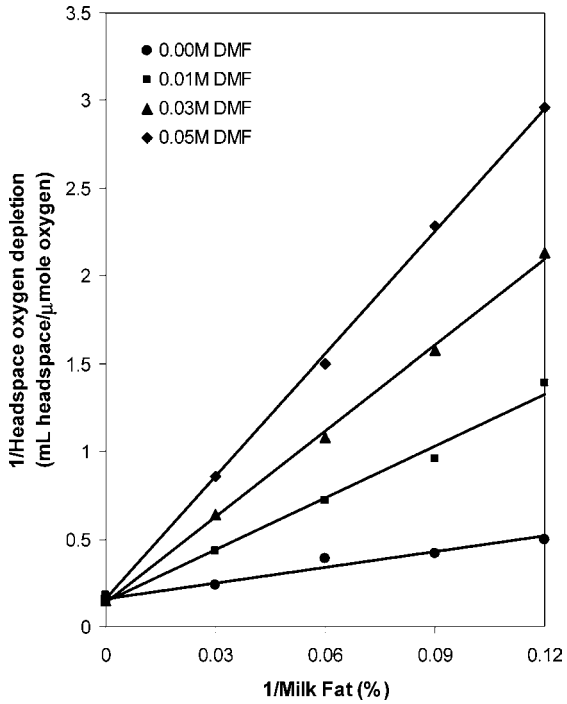

Figure 3. Effect of DMF on the headspace oxygen depletion of milk with milk fat containing 40 μM riboflavin after 3 h of storage under light at room temperature.

Table 2. Parameters in the Plots of $\{-d[O_2]/dt\}^{-1}$ against [Milk Fat]$^{-1}$ of **Figures 2** and **3** for Effects of DABCO and DMF on the Headspace Oxygen Depletion of Milk with 0.21 M Milk Fat Containing 40 μM Riboflavin after 3 h of Storage under Light at Room Temperature

		intercept (mL headspace/ μmol O_2)	slope (M mL headspace/ μmol O_2)	S_Q/S_O[a] (M)
DABCO (M)	0.00	0.144	0.04	1.00
	0.01	0.164	0.09	2.25
	0.03	0.163	0.13	3.25
	0.05	0.172	0.18	4.50
DMF (M)	0.00	0.144	0.04	1.00
	0.01	0.178	0.11	2.75
	0.03	0.151	0.19	4.75
	0.05	0.173	0.28	7.00

[a] S_Q/S_O are the slopes of the plot $\{-d[O_2]/dt\}^{-1}$ against [Milk Fat]$^{-1}$ in the presence and absence of DABCO and DMF, respectively.

S_Q/S_O against [Q] gives a straight line whose slope is $(k_q + k_{ox-Q})/k_d$, from which the rate constant of total quenching $k_q + k_{ox-Q}$ can also be measured when k_d is known.

The results of quenching mechanism study of DABCO and DMF during singlet oxygen oxidation of milk are shown in **Figures 2** and **3**, respectively. The *y*-intercepts of the plot $\{-d[O_2]/dt\}^{-1}$ against $[A]^{-1}$ at 0.01, 0.03, and 0.05 M of DABCO and DMF were statistically the same ($P > 0.05$), but the slopes were different. The steady state kinetic equation states when *y*-intercepts of plots $\{-d[O_2]/dt\}^{-1}$ against [milk fat]$^{-1}$ at different concentrations of quencher are the same, the slopes of the plots are different. The quencher minimizes singlet oxygen oxidation by quenching singlet oxygen only. Therefore, DABCO and DMF quenched singlet oxygen only and did not quench the excited triplet state of riboflavin to minimize the riboflavin photosensitized oxidation of milk.

The slopes and the ratios of S_Q/S_O of the plots containing 0, 0.01, 0.03, and 0.05 M DABCO and DMF calculated from **Figures 2** and **3** are given in **Table 2**. The *y*-intercept and slope of the plot $\{-d[O_2]/dt\}^{-1}$ against $[A]^{-1}$ without quencher were 0.144 mL of headspace gas/μmol of oxygen and 0.04 mL of headspace gas/μmol of oxygen, respectively. The linear regression for the plot of $[AO_2]^{-1}$ against $[A]^{-1}$ without DABCO is $y = 0.04x + 0.144$, where $y = [AO_2]^{-1}$ and $x = [A]^{-1}$. The slope/intercept ratio of the regression line for milk without DABCO is equal to k_d/k_r where k_d is the decaying rate of singlet oxygen in water and k_r is the reaction rate constant of singlet oxygen with milk fat (*35*). The k_d/k_r value was 0.28 M. The k_d singlet oxygen in water was 2.27×10^5 s^{-1} (*37*). Therefore, the reaction rate constant (k_r) of singlet oxygen with milk fat was 2.27×10^5 s^{-1}/0.28 M $= 8.1 \times 10^5$ M^{-1} s^{-1}.

To determine the total singlet oxygen quenching rate $k_q + k_{ox-Q}$ of DABCO, the regression line of S_Q/S_O against [DABCO] was plotted using the data in **Table 2** and is shown in **Figure 4**. k_q and k_{ox-Q} are physical and chemical singlet oxygen quenching rate constants of the quencher, respectively (*36*). S_Q and S_O are the slopes of the plot $\{-d[O_2]/dt\}^{-1}$ against $[A]^{-1}$ in the presence and absence of quencher, respectively. The slope of the regression line of the plot S_Q/S_O against [DABCO] is $(k_q + k_{ox-Q})/k_d$ (*28, 35*). The slope of the regression line of the value of S_Q/S_O against [DABCO] was 66.10 M^{-1}. Because the decaying rate constant of singlet oxygen in water was 2.27×10^5 s^{-1}, the total singlet oxygen quenching rate constant $k_q + k_{ox-Q}$ of DABCO in milk was 1.5×10^7 M^{-1} s^{-1}.

To determine the total singlet oxygen quenching rate $k_q + k_{ox-Q}$ of DMF, the regression line of S_Q/S_O against [DMF] was plotted using the data in **Table 2** and is shown in **Figure 5**. The slope of the regression line of S_Q/S_O against [DMF] was 116.10 M^{-1}. Because the decaying rate constant of singlet oxygen in water was 2.27×10^5 s^{-1}, the total singlet oxygen quenching rate constant $k_q + k_{ox-Q}$ of DMF in milk was 2.6×10^7 M^{-1} s^{-1}. The total quenching rate of DMF was 1.7 times faster than DABCO in milk.

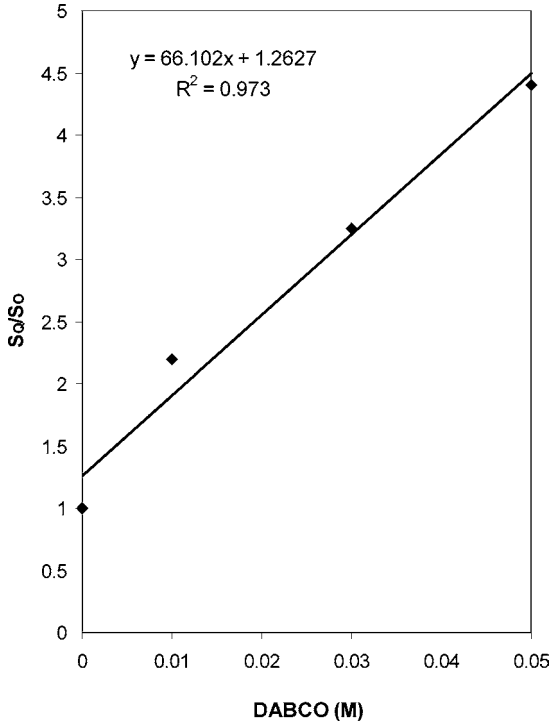

Figure 4. Regression line of S_Q/S_O against concentrations of DABCO in milk.

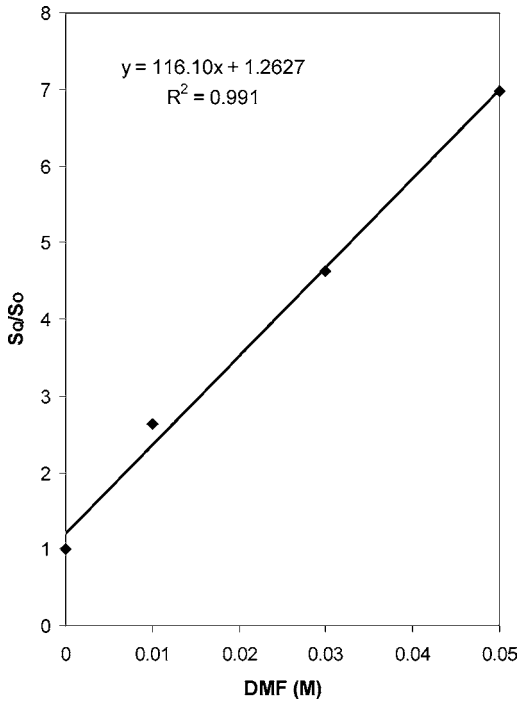

Figure 5. Regression line of S_Q/S_O against concentrations of DMF in milk.

Because the reaction rates of DABCO and DMF with singlet oxygen were extremely high, DABCO and DMF could be used to protect nutrients such as riboflavin and vitamin D_2 in milk

and to improve the oxidative stability of milk fats during storage in dairy products at relatively low concentrations.

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