KINETICS AND EFFECTS OF RIBOFLAVIN PHOTOSENSITIZED DEGRADATION ON SOYMILK FLAVOR STABILITY

DISSERTATION

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By

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ABSTRACT

The kinetics of photosensitized degradation of riboflavin in aqueous solution was studied by the combination of UV-Vis spectrophotoscopy, HPLC, and LC/MS analyses. Lumichrome and lumiflavin were identified as the major photodegradation products from riboflavin exposed to light. The effects of riboflavin and its major photodegradation products, lumichrome and lumiflavin, on the flavor stability of foods were studied using soymilk as a model system.

As one of the water soluble photosensitizers, riboflavin produces singlet oxygen under light. The singlet oxygen formation rate by riboflavin was 2.31 µmole oxygen/mL headspace.h of the serum bottle. The degradations of riboflavin under light after 24 h were 66% in D₂O and 40% in H₂O, respectively. The results indicate that singlet oxygen was involved in riboflavin degradation under light. Ascorbic acid had a protective effect on riboflavin from photodegradation. When 0 mM or 160 mM ascorbic acid was included in riboflavin solution and stored under light for 96 h, the degradation of riboflavin was 94.0% and 15.7%, respectively. Sodium azide reduced the degradation of riboflavin under light, but with a different quenching mechanism. Ascorbic acid quenched both singlet oxygen and excited triplet riboflavin while sodium azide quenched only the singlet...
oxygen in riboflavin solution. The singlet oxygen quenching rate of sodium azide was $1.547 \times 10^7 \text{M}^{-1} \text{s}^{-1}$.

Steady-state kinetic analysis indicated that the reaction rate between riboflavin and singlet oxygen was $1.01 \times 10^{10} \text{M}^{-1} \text{s}^{-1}$. This diffusion-controlled reaction rate explained the rapid degradation of riboflavin in foods under light. Considering the involvement of both the Type-I and Type-II mechanisms in the riboflavin degradation under light, singlet oxygen quencher alone could not protect the riboflavin from degradation completely.

Lumichrome and lumiflavin were identified as the major photodegradation products from riboflavin. The formation of these degradation products was significantly influenced by pH. In neutral or acidic conditions, lumichrome was the only major degradation product; in basic conditions, both lumiflavin and lumichrome were present as degradation products.

As an essential nutrient and effective photosensitizer, riboflavin had profound effect on flavor qualities of foods under light. The headspace oxygen and volatile compounds were determined by gas chromatography for soymilks with or without added riboflavin in serum bottles stored under light or in dark. Riboflavin had significant effects on the headspace oxygen depletion and volatile compounds formation in soymilk under light ($P<0.05$). When the samples were stored in dark, the effects were not significant ($P>0.05$). The volatile compounds increased under light, but not in dark as the added riboflavin increased. Storage temperatures at either $4^\circ\text{C}$ or $20^\circ\text{C}$ did not have significant differences in the effects of riboflavin on the headspace oxygen depletion in soymilk under light. Hexanal, an important “beany” flavor compound, was identified as the major volatile compound in the riboflavin photosensitized soymilk. Hexanal could be formed by
singlet oxygen oxidation. Ascorbic acid, a quencher for singlet oxygen and the excited triplet sensitizers, significantly inhibited the formation of hexanal and total volatiles in soymilk under light.

Considering lumichrome and lumiflavin as the major photodegradation products from riboflavin, and riboflavin as an effective photosensitizer, the next area to investigate was whether the photodegradation products from riboflavin could also function as photosensitizers. Headspace oxygen and headspace volatiles were subsequently analyzed in soymilk samples that had been supplemented with either riboflavin, lumichrome, or lumiflavin. The purpose of such tests was to determine if these degradation products could function as photosensitizers in foods. Similar to riboflavin, lumichrome and lumiflavin caused significant headspace oxygen depletion in soymilk under light. Headspace oxygen depletion was insignificant in soymilk in dark. SPME/GC/FID analysis indicated that soymilk supplemented with riboflavin, lumichrome, or lumiflavin produced much more headspace volatiles under light. The results indicated that riboflavin, lumichrome, and lumiflavin were effective photosensitizers. Ascorbic acid can be added to inhibit the flavor changes in soymilk.
Dedicated to my wife, Rubing Xia,

son, Edward Huang,

daughter, Shawna Huang,

and my parents
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Most importantly, I sincerely thank my wife, Rubing Xia, for her support and taking on the extra duties of caring for the children and other family issues. I appreciate the understanding from my children, Edward and Shawna, for the lack of time spent with them during the course of my study.
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FIELD OF STUDY

Major Field: Food Science and Nutrition
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CHAPTER 1

INTRODUCTION

Riboflavin is a naturally present nutrient in milk, eggs, meat products, and vegetables (Szczesniak and others 1971; Gliszczynska and Koziolowa 1999; USDA 2003). It is also used to fortify processed foods (Singh and others 1996; Chen and others 2001). It is an essential vitamin because the human body has to obtain this compound from the diet. Riboflavin is an active part of the coenzymes of flavin mononucleotide (FMN) and flavin-adenine dinucleotide (FAD). These coenzymes have essential roles in several dehydrogenase and oxidase activities. Riboflavin has a complex photochemistry due to its ability to accept or lose a pair of hydrogen atoms (Isaka 1955; Kim and others 1993; Crank and Pardijanto 1995; Li and Min 1998a; Edwards and others 1999; Criado and others 2003; Viteri and others 2003). Riboflavin under light can generate reactive oxygen species such as superoxide anions and singlet oxygen (Mahns and others 2003). Formation of reactive oxygen species by riboflavin under light proceeds by both the Type-I and Type-II mechanisms (de La Rochette and others 2003). After receiving energy from light, riboflavin converts to excited triplet riboflavin. In the Type-I mechanism, the excited triplet riboflavin is reduced by abstraction of electrons or
hydrogen ion from other food components to form riboflavin radical. In the Type-II mechanism, the excited triplet riboflavin reacts with atmosphere triplet oxygen to produce superoxide anion, and singlet oxygen (Foote 1985). 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 (Kumari and others 1996; Min and Boff 2002; Mahns and others 2003).

Reactive oxygen species can cause not only the nutrient’s destruction, but also an off-flavor formation in foods. While it is relatively stable during food processing and storage in dark, riboflavin is very sensitive to UV and visible light. Thirty percent of the riboflavin in milk was lost after exposure to sunlight for 30 min (Wishner 1964). Twenty to thirty percent of the riboflavin was degraded under light with wavelength between 290 and 400 nm (Joshi 1989). It has been reported that the decomposition products from riboflavin in milk under light were lumiflavin and lumichrome (Toyosaki and Hayashi 1993). Riboflavin was degraded to lumichrome with only a few minutes of exposure to simulated sunlight. (Tatsumi and others 1992).

Singlet oxygen was suggested to be involved in riboflavin degradation under light (Lee and others 1998). Although many studies on the photosensitization and degradation of riboflavin have been published, the detailed chemical mechanisms and kinetics have not been reported.

Soymilk is a traditional nutritious beverage used for thousands of years in oriental countries. In recent years, soymilk consumption among Western consumers has considerably increased due to heightened awareness of the health benefits of soybean
products. Soy beverages were a $622 million market in 2003, with projections for 2010 at $1.78 billion, and a compounding annual growth rate of 17% in the U.S (Savitry and Prakash 2004). However, due to the association of a beany flavor with soymilk, the acceptance of soymilk in Western countries, including the U.S., is still hampered.

Hexanal is one of the major off-flavor compounds in soy products (Torres-Penaranda and Reitmeier 2001). Hexanal is a product from lipid oxidation, which can be intensified by the exposure of light, pro-oxidant metals (Marsili 1999), or enzymes such as lipoxygenase and hydroperoxylyase (Matoba and others 1985). Hexanal and pentanal were reported to be responsible for the cardboard-like flavors in milk (Hansen and Skibsted 2000; Kristensen and others 2001).

The oxidation of food components can be caused either by diradical triplet oxygen or non-radical singlet oxygen. Diradical triplet oxygen ($^3$O$_2$) readily reacts with radical compounds in foods and is temperature dependent (Frankel 1985). Non-radical singlet oxygen ($^1$O$_2$) reacts directly with electron-rich compounds such as those containing double bonds. Singlet oxygen oxidation is temperature independent. Even at low temperatures, the oxidation rate of food components involving singlet oxygen is rather high (Min and others 1989). Linoleic acid oxidation caused by singlet oxygen is over 1450 times greater than that of triplet oxygen autoxidation (Rawls and VanSanten 1970; Frankel and others 1981).

The most important mechanism for the formation of singlet oxygen in foods is by photosensitized generation. Riboflavin, chlorophyll, myoglobin, and some artificial food colorants are effective photosensitizers (Foote and Denny 1968; King and Min 2002). In the presence of light and molecular oxygen, photosensitizers can generate singlet oxygen.
One molecule of sensitizer may generate $10^3$ to $10^5$ molecules of singlet oxygen before becoming inactive (Min and others 1989; Kochevar and Redmond 2000).

Riboflavin is a water soluble photosensitizer (Min and Boff 2002). Upon absorbing energy from UV or visible light, riboflavin becomes an excited singlet state. An excited triplet state of riboflavin is formed from the excited singlet state through the intersystem crossing mechanism. The excited triplet state riboflavin reacts with triplet molecular oxygen to form singlet oxygen (the Type-II mechanism). The excited triplet riboflavin has a redox potential of 1.7 V at pH 7 (Lu and others 1999). It can abstract electron or hydrogen atom from other substrates in foods such as polyunsaturated fatty acids and form free radicals of food compounds (the Type-I mechanism).

Soymilk contains 2.86% protein, 1.53% fat, 0.27% ash, 1.53% carbohydrate, and 93.81% of moisture (Rosenthal and others 2003). Riboflavin content in soymilk is about 3 ppm, which is comparable to the riboflavin content in cows milk (Kwok and others 1998). The effect of riboflavin on the formation of off-flavors in cow milk under light storage was thoroughly studied (Lee 2002). Pentanal, hexanal, heptanal, and dimethyl disulfide were found to increase significantly when milk was stored under light in the presence of increased levels of riboflavin.

Many attempts have been made to eliminate or minimize the beany flavor in soy products. Those include masking beany flavor with flavor materials (Lee and others 1990), process modification to reduce or eliminate beany flavors in soy products (Hunter and others 1983; Gupta 2003), and genetic modification of soybean varieties to reduce the enzymes responsible for the beany flavors (Ma and others 2001; Feng and others 2001). However, few studies were reported on the flavor changes of soymilk during
storage under retail supermarket conditions. The effects of riboflavin in soymilk on the formation of volatile compounds under light were not reported.

The objectives of this dissertation are (1) to prove the involvement of single oxygen in riboflavin degradation under light, (2) to identify the major photogradation products from riboflavin, (3) to determine the reaction rate between singlet oxygen and riboflavin, lumichrome, or lumiflavin, (4) to study the effects of ascorbic acid and sodium azide on the riboflavin degradation under light and their reaction mechanisms, (5) to study the effect of riboflavin and its degradation products on the formation of volatile compounds in soymilk under light, and (6) to determine the effects of ascorbic acid, a quencher for both the singlet oxygen and excited triplet riboflavin, on the formation of beany flavor compounds in soymilk under light.
CHAPTER 2

LITERATURE REVIEW

2.1 Chemistry of oxygen

The oxidation of food components has long been classified as a major deterioration process affecting the sensory and nutritional qualities of foods. Oxidation can be caused by either triplet oxygen or singlet oxygen. While the understanding of ground triplet state oxygen dates back 200 years, the discovery of the existence of singlet oxygen only had 70 years (Min and Boff 2002). Singlet oxygen ($^1O_2$) is in the lowest electronic excited state just above the ground triplet state ($^3O_2$). Because oxygen in this activated metastable state is much more reactive than the ground triplet state, $^1O_2$ becomes one of the main reactive oxygen species responsible for the biological and biochemical damages under light. The relative reaction rates of triplet oxygen and singlet oxygen with major fatty acids in oils are shown in Table 1 (Gunston 1986). The reaction rates of singlet oxygen with various fatty acids are 1,000 to 30,000 times faster than that of triplet oxygen. The reaction rates of triplet oxygen and singlet oxygen with linoleic
acid were estimated to be $8.9 \times 10^1 \, M^{-1}s^{-1}$ and $1.3 \times 10^5 \, M^{-1}s^{-1}$, respectively (Rawls and VanSanten 1970). Therefore, the reaction rate of singlet oxygen with linoleic acid is about 1450 times faster than that of the triplet oxygen with linoleic acid.

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<td>27</td>
<td>77</td>
</tr>
<tr>
<td>$^1O_2$</td>
<td>$3 \times 10^4$</td>
<td>$4 \times 10^4$</td>
<td>$7 \times 10^4$</td>
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(Gunston 1986)

Table 1  Relative oxidation rates of triplet oxygen and singlet oxygen with oleate, linoleate, and linolenate.

2.1.1  The formation of singlet oxygen

As shown in Figure 1, singlet oxygen may be formed by chemical, enzymatic, photosensitization, and physical methods (Krinsky 1977; Min and Boff 2002). In food and other biological systems, the most common means of singlet oxygen generation is
photosensitization. Riboflavin as a water soluble food component and chlorophyll as an oil soluble food component are well known to be the efficient sensitizers for singlet oxygen formation in foods.

Figure 1  Formation of singlet oxygen by chemical, photochemical, and biological methods.

Sensitizer, molecular oxygen, and energy from light are three essential elements for the photosensitized generation of single oxygen. Light promotes sensitizer (Sen) to
an excited singlet state ($^1\text{Sen}^*$). With the intersystem crossing mechanism, $^1\text{Sen}^*$ subsequently becomes the excited triplet state ($^3\text{Sen}^*$), which has a much longer lifetime to react with ground state triplet oxygen ($^3\text{O}_2$). The interaction between $^3\text{Sen}^*$ and $^3\text{O}_2$ can result in the generation of $^1\text{O}_2$ (Figure 2).

![Diagram of singlet oxygen formation](image)

Figure 2 Photosensitized formation of singlet oxygen.

The energy of singlet oxygen is 22.5 kcal/mole above the ground state triplet oxygen. It is non-radical in nature and reacts with non-radical, singlet-state, and electron-rich compounds such as those containing double bonds. The most common reaction types of singlet oxygen with olefins are 1,2-cycloaddition, 1,4-cycloaddition, and “ene” reactions (Figure 3).
Figure 3  Reaction of singlet oxygen with olefins through 1,2-cycloaddition, 1,4-cycloaddition, and "ene" reaction.
2.1.2 Type-I and Type-II mechanisms

In photosensitized systems, oxidative reactions proceed through either a Type-I or a Type-II mechanism. In a Type-I mechanism, the excited triplet sensitizer reacts directly with compound to produce free radicals or free radical ions (Gollnick 1968; Foote 1976; Sharman and others 2000). In a Type-II mechanism, the excited triplet sensitizer reacts with $^3\text{O}_2$ to form singlet oxygen ($^1\text{O}_2$) and singlet sensitizer through triplet sensitizer-triplet oxygen annihilation. Energy is transferred from the high energy excited triplet sensitizer to low energy triplet oxygen to form high energy singlet oxygen and low energy ground state singlet sensitizer (Sharman and others 2000). More than 99% of the reaction between triplet sensitizer and triplet oxygen produces singlet oxygen (Kepka and Grossweiner 1972). A small amount of such a reaction produces superoxide anion (Kepka and Grossweiner 1972).

A number of factors influence Type-I and Type-II mechanisms during photosensitized oxidative reactions. The availability of triplet oxygen in the reaction environment is one of the most important factors. Higher oxygen availability favors the Type-II reaction. Since oxygen has higher solubility in oil and other non-polar solvent systems, the Type-II mechanism is more predominant than Type-I in an oil system. Likewise, Type-I is likely more predominant in an aqueous system due to the limited solubility of triplet oxygen.

The competition of reaction substrates for excited triplet sensitizer also determines whether the reaction favors a Type-I or a Type-II mechanism. At a higher substrate
concentration, the chance of the excited triplet sensitizer reacting with triplet oxygen is reduced; therefore, the Type-I mechanism is predominate.

2.1.3 Detection of singlet oxygen

To characterize photosensitized oxidation as Type-I, Type-II, or both, the detection of singlet oxygen is a necessary research tool. Since the lifetime of singlet oxygen in solution and solid samples ranges from nano- to micro-seconds, direct measurement of singlet oxygen has been difficult.

The time-resolved detection method has been one of the standard techniques for the determination of singlet oxygen formation yield, lifetime, and deactivation rate constants (Schweitzer and Schmidt 2003). However, because of the limitations in the sensitivity of cryogenic germanium diodes, this method is insufficient in providing quantitative information for aqueous media and biological systems.

The application of trapping molecules is a necessary tool to indirectly detect the presence of singlet oxygen. 2,2,6,6-Tetramethyl-4-piperidone (TMPD) can react with singlet oxygen to produce the corresponding nitroxide, 2,2,6,6-tetramethyl-4-piperidone-1-oxyl (TAN). TAN can be detected by ESR spectroscopy. The formation of TAN was detected in 5 mM riboflavin in phosphate buffer, and in skim milk containing 20 mM TMPD during 10 minutes of illumination under fluorescent light (Bradley and others 2003). These studies indicate that the formation of singlet oxygen requires the presence of each of these components, i.e., the light, the sensitizer riboflavin, and the oxygen. The
formed TAN can be further reduced to the hydroxylamine of TAN, thus decreasing the paramagnetic TAN signal. The maximum concentration of TAN was obtained with 10 mM of riboflavin added in either buffer solution or in skim milk after 15 min of illumination in the presence of oxygen (Bradley and others 2003).

Future methods will increase sensitivity by using a non-fluorescent substrate that will fluoresce upon the reaction with singlet oxygen or will increase the fluorescence signal upon the energy transfer from the singlet oxygen (Schweitzer and Schmidt 2003).

2.1.4 Quenchers of singlet oxygen and quenching mechanisms

Quenchers typically have highly conjugated unsaturated structures. α-tocopherol and β-carotene are effective singlet oxygen quenchers in food systems. One molecule of β-carotene is estimated to quench up to 1000 molecules of singlet oxygen (Foote and others 1970).

A schematic diagram for single oxygen-related oxidation is shown in Figure 4. To minimize singlet oxygen oxidation, quenchers may interact with the excited triplet sensitizer (\(^3\text{Sen}^*\)) or the singlet oxygen (\(^1\text{O}_2\)). In the first case, the excited triplet sensitizer (\(^3\text{Sen}^*\)) interacts with a quencher (Q) to become ground-state (Sen), resulting in less formation of singlet oxygen (\(^1\text{O}_2\)). In the second case, quenchers interact either physically or chemically with singlet oxygen (\(^1\text{O}_2\)), resulting in less singlet oxygen (\(^1\text{O}_2\)) reacting with valuable food components (Min and Boff 2002).
The steady-state kinetic equation for singlet oxygen quenching and the excited sensitizer is as follows (Foote 1979; Yang and others 2002):

\[
\frac{d[AO_2]}{dt} = K^{-1}(1 + \frac{k_Q[Q]}{k_o[3O_2]})(1 + \frac{(k_q + k_{ox-Q})[Q]}{k_d} \times [A]^{-1})
\]

where \([AO_2]\) is the concentration of oxidized substrate; \(K\) is the quantum yield of intersystem crossing; \(k_Q\) is the reaction constant for triplet sensitizer quenching; \([Q]\) is the quencher concentration; \(k_o\) is the reaction constant for singlet formation by energy transfer from excited triplet sensitizer to triplet oxygen; \([3O_2]\) is the triplet oxygen concentration; \(k_q\) is the reaction constant of singlet oxygen physical quenching; \(k_{ox-Q}\) is the reaction constant of singlet oxygen chemical quenching; \(k_d\) is the reaction rate for
singlet oxygen decay in a solvent; $k_r$ is the reaction constant between substrate and singlet oxygen; and $[A]$ is the initial concentration of substrate.

When only the singlet oxygen quenching is involved in the reaction, the equation can be simplified as follows:

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

Plots of $[AO_2]^{-1}$ against $[A]^{-1}$ at various concentrations of quencher will result in similar $y$-intercepts and different slopes (Figure 5). The intercept of this plot would be equal to $K^{-1}$ and the slope would be equal to $K^{-1} \times \frac{(k_q + k_{as-Q})[Q] + k_d}{k_r}$. The ratio of the slope to intercept (S/I) would become $\frac{(k_q + k_{as-Q})[Q] + k_d}{k_r}$, which is independent of $O_2$ concentration. A new graph S/I plotted against $[Q]$ as shown in Figure 6 would result in a line with an intercept of $k_d/k_r$ and a slope of $k_q + k_{as-Q}/k_r$.

If there is only excited triplet riboflavin quenching, then the equation becomes:

$$\left\{\frac{d[AO_2]}{dt}\right\}^{-1} = K^{-1} \left(1 + \frac{k_Q[O]}{k_o[3O_2]}\right)(1 + \frac{k_d}{k_r} \times [A]^{-1})$$

Both the $y$-intercept and the slope in the plot of $[AO_2]^{-1}$ against $[A]^{-1}$ will vary according to the concentration of a quencher acting on the excited triplet sensitizer. The $y$-intercept would be equal to $K^{-1} \left(\frac{k_o[3O_2] + k_Q[O]}{k_o[3O_2]}\right)$ and the slope would be equal to $K^{-1} \left\{\frac{k_d(k_o[3O_2] + k_Q[O])}{k_o[3O_2]}\right\}$. The ratio of the slope to intercepts, $\frac{k_d}{k_r}$, would be the same regardless of the quencher concentration.
Figure 5 Quenching mechanism of singlet oxygen.
Figure 6  Plot of slope/intercept of regression line from Figure 5 vs. quencher concentrations.

$$\text{Intercept} = \frac{K_d}{K_r}$$

$$\text{Slope} = \frac{(K_q+K_{ox}-Q)}{K_r}$$
Figure 7  Quenching mechanism of excited triplet sensitizer.
When there are both excited triplet sensitizer quenching and singlet oxygen quenching, both the y-intercept and the slope in the plot of $[\text{AO}_2]^{-1}$ against $[A]^{-1}$ will vary as in Figure 7. The y-intercept would be equal to $K^{-1}(\frac{k_d[\text{O}_2] + k_q[\mathcal{Q}]}{k_d[\text{O}_2]})$ and the slope would be equal to $K^{-1}\left\{\frac{(k_d[\text{O}_2] + k_q[\mathcal{Q}])((k_q + k_{\text{ox}-\mathcal{Q}})[\mathcal{Q}] + k_d)}{k_r k_d[\text{O}_2]}\right\}$. The ratio of the slope to y-intercept would be $\frac{(k_q + k_{\text{ox}-\mathcal{Q}})[\mathcal{Q}] + k_d}{k_r}$. All of these terms vary with the quencher concentrations.

When there is no quencher added in an experimental system, the equation can be further simplified to the following (Foote 1979; Jung and others 1995; Lee and others 1997):

$$\left\{\frac{d[\text{AO}_2]}{dt}\right\}^{-1} = K^{-1}(1 + \frac{k_d}{k_r} \times [A]^{-1})$$

When plotting $[\text{AO}_2]^{-1}$ against $[A]^{-1}$, the ratio of the slope to the intercept of the plot is $k_d/k_r$. If the singlet oxygen decay rate, $k_d$, is known, it will be possible to determine the reaction rate constant, $k_r$, between the substrate and singlet oxygen (Foote 1979).
2.2 Chemical reactions and stability of riboflavin in foods

Riboflavin (vitamin B₂) is a yellowish compound present in most living organisms. Riboflavin was first isolated from whey over 100 years ago. Riboflavin and flavin adenine dinucleotide were isolated from brewers’ yeast (Warburg and Christian 1932). It was suggested that riboflavin played an important role in cell respiration. The first pure riboflavin was synthesized about 70 years ago (Kuhn and others 1935). Riboflavin is now being produced commercially by both chemical synthesis and microbial fermentation (Bretzel and others 1999; Schwogler and others 2000; Szczesniak and others 1971). Of 3,000 tons of riboflavin produced annually, 2,500 tons are produced by the microbial fermentation method.

Riboflavin is an active part of the coenzymes of flavin mononucleotide and flavin adenine dinucleotide that catalyze many oxidation-reduction reactions. These coenzymes have essential roles in several dehydrogenases and oxidases. Riboflavin can accept or donate a pair of hydrogen atoms. Riboflavin has complex photochemistry since it is easily reduced and oxidized by accepting and donating hydrogen or electron.

When irradiated with visible or UV light, riboflavin can produce reactive oxygen species such as superoxide anion radicals, singlet oxygen, hydroxyl radical, and hydrogen peroxide in the presence of atmospheric oxygen. The distribution of reactive oxygen species in a particular riboflavin photosensitized system depends on the availability of oxygen, the concentration of riboflavin and other oxidizable substrates, and the presence of quenchers. Reactive oxygen species can cause not only the destruction of protein,
carbohydrates, lipids, and vitamins, but also the formation of off-flavor and loss of nutrients in foods (Huk and others 1998; Levine 2002; Min and Choe 2002; Lee and others 2003).

2.2.1 Chemistry and biological functions of riboflavin

Riboflavin has many conjugated double bonds and nitrogens in the ring structure, as shown in Figure 8. Riboflavin shows absorption maxima at 225, 275, 370, and 450 nm at pH 7 (Drossler and others 2002) and exists in cationic form (RFH$_2^+$) at low pH (<4.0), neutral form (RFH), and anionic form (RF$^-$) at high pH (>9.7).

![Structure of riboflavin](image)

Figure 8 Structure of riboflavin.
The cationic riboflavin is non-fluorescent, the neutral form is fluorescent, and the anionic form is weakly fluorescent (Drossler and others 2002). The fluorescence quantum yield and lifetime of riboflavin depend on the solvent, pH, and presence of amino acids in the system (Drossler and others 2002). Riboflavin in organic protic solvent such as methanol gives a higher fluorescence quantum yield and longer lifetime than that in sulfur containing organic solvent (e.g., dimethylsulfoxide), or in organic protic solvent (e.g., water). The fluorescence of riboflavin is quenched by sulfur containing amino acids, such as methionine and cystein (Drossler and others 2002). The lifetime of riboflavin decreases as the concentration of methionine and cystein increases. Methionine quenches riboflavin fluorescence by forming a riboflavin anion-methionine cation pair in the ground state and excited state; this quenching is pH independent in the range of 5.2-9.0. The riboflavin fluorescence quenching by cystein increases as pH increases. Cystein, present in thiolate form (RS\textsuperscript{-}) at high pH, reacts with neutral riboflavin. The riboflavin is deprotonated and produces riboflavin anion and cystein in a thiol form RSH (Drossler and others 2002).

Riboflavin is essential for overall normal growth and development of the body, production and regulation of certain hormones, and formation of red blood cells (Ajayi and others 1993). Riboflavin helps cells to metabolize carbohydrates, lipids, and proteins and is crucial for the production of biological energy in the electron transport system. It is also necessary for the maintenance of healthy vision, skin, hair, and nails. Riboflavin is involved in the utilization of neurotransmitters, which are implicated in emotional health including the development of depression. Deficiency of riboflavin is mainly manifested in the skin and mucous membranes with the following symptoms: cracks and
sores in the corners of the mouth, lesions of the lips, and a red sore tongue. Visual disturbances including a gritty feeling on the insides of the eyelids, burning and fatigue of the eyes, dilation of the pupils, changes in the cornea, and sensitivity to light may be indicative of riboflavin deficiency. Sometimes cataracts result. Riboflavin should be consumed in the diet since it is not stored in the body. The recommended daily allowance of riboflavin is between 1.3 and 1.7 mg among different age groups; milk, eggs, leaf vegetables, and soybeans are excellent sources of riboflavin. Riboflavin content in 100 g of each of the following egg products were as follows: 1154 µg in egg powder, 310 µg in raw egg white, and 352 µg in raw egg yolk (Gliszczynska-Swiglo and Koziolowa 2000). Various milk products have riboflavin contents ranging from 100 µg to 180 µg/100 g (USDA 2003).

2.2.2 Stabilities of riboflavin

2.2.2.1 Processing effect

Riboflavin is relatively stable during processing foods to increase the shelf life, such as dehydration or γ-irradiation (Farrell and Fellers 1942). Riboflavin in dehydrated fruits and vegetables was quite stable even after being stored at 54°C for one year. When dehydrated foods were packed in metal containers with air, N₂, or CO₂, and in paper cartons for one year at 54°C, riboflavin was stable while carotene, ascorbic acid, and
thiamin were not (Heberlein and Clifcorn 1944). Riboflavin in milk powder was relatively stable at 60°C while folic acid, thiamin, vitamin B₆, and pantothenic acid were rapidly destroyed.

Riboflavin in cod fish fillets was reduced 6% by irradiation and 9% by cooking (Kennedy and Ley 1971). Riboflavin in prawns did not significantly change after γ-irradiation up to 7 KGy, even after post-irradiation heating at 100°C for 10 min (Lee and Hau 1996). Riboflavin is normally bound to proteins which protect the prosthetic groups from being attacked directly or indirectly by γ-irradiation. The level of riboflavin remains largely unchanged in most foods stored for 18 months at 37°C (Brenner and others 1948).

Riboflavin stability is affected by oxygen, water activity, and other components. When oxygen is present during storage, destruction rate of riboflavin increases dramatically (Dennison and others 1977). When riboflavin is mixed with metal sulphates, riboflavin is significantly (P<0.01) deteriorated during 90-180 day storage (Marchetti and others 2000). Riboflavin stability was not affected by storage temperature in the presence of amino acid chelate. Water activity (A_w) plays an important role in riboflavin stability in a low-moisture dehydrated model food system. Riboflavin retention at A_w 0.1-0.65 at 20 °C and A_w 0.1-0.40 at 30°C was almost 100% after 8 months. Riboflavin loss increased with increasing A_w (Dennison and others 1977).

2.2.2.2 Heat effect

The heating process barely affects the riboflavin in foods. High amounts of riboflavin remained in roasted pork despite a heating process which significantly
destroyed other vitamins (Lassen and others 2002). Studies have shown that riboflavin retention was independent of heating methods such as hot air convection, infrared, high-pressure steam, and microwave (Ang and others 1975). Riboflavin is more heat stable and less temperature sensitive than thiamin (Kwok and others 1998). Thermal degradation of riboflavin in soymilk followed the first order and the reaction rate constants were $7.05 \times 10^{-4}$, $4.26 \times 10^{-3}$, and $2.12 \times 10^{-2}$/min at 90, 120, and 140°C, respectively. The D values of riboflavin at 90, 120, and 140°C were 3268, 540, and 109 min, respectively; the Z value was 36°C (Kwok and others 1998).

2.2.2.3 Light effect

While riboflavin is relatively heat-stable, it is very sensitive to light. Milk lost 30% riboflavin to sunlight exposure and only 12% riboflavin to boiling for 30 min (Wishner 1964). Riboflavin photodegradation in dried macaroni and nonfat dried milk occurred via a two-phase mechanism (Furuya and others 1984). A two-phase mechanism includes an initial rapid degradation phase followed by a second, slow loss phase of riboflavin under light. Both phases demonstrate first order degradation. Riboflavin photodegradation followed the first order degradation mechanism in liquid systems such as skim milk and a buffered solution of riboflavin. Riboflavin loss under light was dependent on the light intensity, exposure time, light wavelength, packaging materials, and food processing methods. Sunlight was more detrimental to riboflavin than fluorescent light. Light at 450 nm, the maximum absorption wavelength of riboflavin, was the most destructive to riboflavin. Milk stored in a clear bottle or white sachet lost
riboflavin faster than milk stored in a brown bottle or carton (Satter and deMan 1975; Bekbolet 1990). Heat treatment and homogenization of milk increased the photostability of riboflavin in milk by light absorption or scattering due to the changing some of the physico-chemical properties of milk (Saidi and Warthesen 1995).

Riboflavin in cheese decreased to 25% of the original content after 100 days of storage under light (Wold and others 2002). The content of riboflavin inside the cheese was higher than that on the cheese surface. Riboflavin on the surface of cheese blocks was almost completely destroyed after one month under light. Riboflavin content inside the cheese blocks changed only slightly after one month under light.

2.2.3 Riboflavin as a photosensitizer and reactive oxygen species formation

Riboflavin is a well-known photosensitizer. The excitation and deactivation of riboflavin under light is shown in Figure 9.

Excited triplet riboflavin, which is diradical, reacts with triplet oxygen having unpaired electrons and produces superoxide anion by electron transfer or singlet oxygen by energy transfer (Min and Boff 2002), which is called Type-II photosensitization (Figure 10). The reaction rate of triplet state riboflavin with triplet oxygen is $9 \times 10^8 \text{M}^{-1}\text{s}^{-1}$ (Lu and others 2000). Formation of a superoxide anion by the interaction of triplet riboflavin and triplet oxygen is very low (Kepka and Grossweiner 1972). Bradley and others (2003) proved the formation of singlet oxygen by riboflavin under light by electron
spin resonance spectroscopy. Lumiflavin and lumichrome produce singlet oxygen at 320-400 nm with 15% higher and 60% lower, respectively, than riboflavin (Joshi 1989).

Figure 9  Excitation and deactivation of riboflavin under light.

Triplet riboflavin is frequently reduced by the abstraction of electrons or a hydrogen atom from food components (R’H) and anionic riboflavin radical (RF•) and reduced riboflavin radical (RFH₂•), which is called the radical or Type-I photosensitization mechanism.
Reactive oxygen species such as singlet oxygen, superoxide anion, hydroxyl radical, and hydrogen peroxide are formed in the presence of riboflavin under light (Jernigan 1985; Naseem and others 1993; Bradley and Min 1992). A superoxide anion can form hydrogen peroxide by dismutation. The reaction of a superoxide anion and hydrogen peroxide produce a hydroxyl radical by the Haber-Weiss reaction (Haseloff and Ebert 1989).

\[
\begin{align*}
O_2^- &+ O_2^- \xrightarrow{\text{Dismutation}} H_2O_2 + ^1O_2 \\
H_2O_2 &+ O_2^- \xrightarrow{\text{Haber-Weiss reaction}} O_2 + OH^- + HO^-
\end{align*}
\]
2.2.4 Mechanisms for the light sensitivity of riboflavin

The light sensitivity of riboflavin has been extensively studied and well known for more than 60 years. The detailed chemical mechanisms for the light sensitivity of riboflavin have not been discussed. The hydroxyl radical, superoxide anion, singlet oxygen, riboflavin cationic, and anionic radicals that are formed by photosensitized riboflavin can be involved in the destruction of riboflavin in foods.

A hydroxyl radical, that has reduction potential of 2300 mV, is one of the strongest oxidizing agents among the reactive oxygen species in foods. A hydroxyl radical is also a strong electrophilic molecule and can easily react with food compounds containing double bonds. This reaction is diffusion controlled with a rate of $5.0 \times 10^9$ or $10^{10} \text{M}^{-1}\text{s}^{-1}$ (Hoffman and Hayon 1973; Solar and others 1984; Motohashi and Saito 1993; Zhao and others 1994). The reaction rate of riboflavin with a hydroxyl radical is $1.2 \times 10^{10} \text{M}^{-1}\text{s}^{-1}$ (Kishore and others 1991).

Singlet oxygen formed by photosensitized riboflavin can be involved in riboflavin degradation in foods. Riboflavin, with its many double bonds, can directly react with electrophilic singlet oxygen (Allen and Parks 1979). The reaction rate of riboflavin with singlet oxygen is on the order of $10^7 \text{M}^{-1}\text{s}^{-1}$ (Chacon and others 1998). Sodium azide, a singlet oxygen quencher, decreases the riboflavin sensitized oxidation of riboflavin (Lee and others 1998). Storage temperature does not affect riboflavin photodegradation, suggesting that riboflavin degradation is due to the singlet oxygen oxidation (Kristensen and others 2001). Riboflavin is photodegraded more rapidly in D$_2$O than in H$_2$O, which
indicates that singlet oxygen is involved in the degradation of riboflavin under light (Huang and others 2004).

When excited triplet riboflavin reacts with other excited or ground state riboflavin molecules, riboflavin cationic (RFH⁺) and anionic radicals (RF⁻) are produced as shown in Figure 11. This one-electron oxidation of riboflavin occurs at the C-8 methyl group and at the extended π–electron system (Lu and others 1999). The radicals formed can react with triplet oxygen and the electron transfer caused by RFH or RFH₂ may be important in the photochemistry and destruction of riboflavin.

\[
\text{RFH} + \text{O}_2 \rightarrow \text{Oxidized riboflavin}
\]

The excited diradical triplet state riboflavin can be converted to deuteroflavin by oxidation, or form a N₁-N₁₀ bridged leucodeuteroflavin by proton exchange as shown in Figure 12.

Figure 11 Formation of riboflavin cationic and anionic radicals.
The polyhydroxy-containing ribityl group in riboflavin is easily cleaved under light (Wu and others 2001). Fragmentation between N 10 and C 1’ in excited triplet riboflavin produces lumichrome and lumiflavin upon exposure to sunlight and ultraviolet light as shown in Figure 13 (Allen and Parks 1979; Joshi 1989; Cui and others 2001). Riboflavin transforms mainly to lumichrome through the removal of the ribityl group and reconfiguration of a double bond in the ring structure in neutral and acidic solutions. The triplet riboflavin transforms to lumiflavin in alkaline solution (Pan and others 2001). Lumichrome and lumiflavin are stable under UV irradiation (Wu and others 2001). Photoreduction and photodealkylation occur predominantly through the excited triplet state of riboflavin.

Figure 12  Deuteroflavin and leucodeuteroflavin formation from triplet state riboflavin.
Figure 13  Lumichrome and lumiflavin formation from triplet riboflavin by dealkylation.
2.2.5 Riboflavin photosensitized oxidation of food components

Triplet riboflavin has a higher reduction potential than amino acids, proteins, lipids, and many vitamins, and can induce food components to photodegrade (Silva and others 1998; Lu and others 2000). The reduction potentials of triplet riboflavin and electron-deficient cation radical of riboflavin (RFH·+) are 1.7 V and 2.28 V, respectively (Lu and others 1999). These riboflavin radicals are very strong oxidizing species.

2.2.5.1 Amino acids and proteins

Aliphatic amino acids produce carbon dioxide and an aldehyde by riboflavin-photosensitized oxidation (Figure 14). This reaction is very similar to the Strecker degradation reaction of an amino acid.

![Figure 14 Oxidation of aliphatic amino acids photosensitized by riboflavin.](image-url)
Tyrosine and tryptophane in distilled water are oxidized in the presence of riboflavin at $7.5 \times 10^{-6}$ M under light. The reduction potential of triplet riboflavin, tyrosine, and tryptophan is 1.70, 0.93, and 1.01 V, respectively (Lu and Liu 2002). At a low oxygen concentration of 5%, excited triplet riboflavin reacts with tyrosine (TyrOH) and produces riboflavin radicals ($RFH_2^\cdot$) and oxidized radicals of tyrosine (TyrO•) via electron transfer by Type-I mechanism (Garcia and Silva 1997; Viteri and others 2003).

$$3RFH^* + TyrOH \rightarrow RFH^- + TyrOH^+$$

$$RFH^- + TyrOH^+ \rightarrow RFH_2^- + TyrO^-$$

Photooxidation of tryptophan in the presence of riboflavin follows both Type-I and Type-II pathways. The Type-II mechanism is favored in the riboflavin sensitized oxidation of tryptophan at high oxygen concentrations under light. Hydroxyl radicals and singlet oxygen were responsible for tryptophan photooxidation by riboflavin. Tryptophan, tyrosine, and histidine have electron rich double bonds and are excellent reactants for singlet oxygen oxidation.

The main photoproduct of tyrosine is bityrosine; for tryptophan the products are indole, flavin, and indole-flavin type aggregates. The reaction rate of riboflavin-photosensitized tryptophan oxidation is $4.19 \pm 1.85 \times 10^{-16}$ M s$^{-1}$ mm$^{-2}$ (de La Rochette and others 2003). The tryptophan degradation by riboflavin photosensitization increases with an oxygen concentration up to 40 µM, and then decreases as the oxygen concentration continues to increase (de La Rochette and others 2000). Tyrosine and tryptophan are also degraded in the presence of riboflavin during illumination under nitrogen. Ascorbic acid reduces the tryptophan photooxidation by interacting with the excited triplet riboflavin (Garcia and Silva 1997).
Peptides containing tryptophan are oxidized rapidly at pH 7.5 in the presence of 21 µM riboflavin and fluorescent light, mostly by singlet oxygen. The oxidation rate is higher when tryptophan is bound on the carboxyl side in dipeptide than when it is bound to the amino side. For a tripeptide containing tryptophan, the oxidation rate is the highest when tryptophan is on the carboxyl side and the lowest when tryptophan is in the middle of a tripeptide (Kanner and Fennema 1987).

Histidine and methionine in distilled water are photooxidized by visible light in the presence of riboflavin at 7.5 x 10^{-6} M by the Type-II mechanism (Paine and Francis 1980). Aspartame, an artificial sweetener and methyl ester of the dipeptide aspartyl phenylalanine, is destroyed by riboflavin and light (Kim and others 1997). The photosensitizing activity of riboflavin for aspartame destruction is pH dependent; aspartame destruction is high at a pH of 7, but is not significant at a pH of 4 or 6.

Collagen is crosslinked by riboflavin photosensitized reactions (Kato and others 1994). The aggregation of collagen due to cross linkage is accompanied by the loss of tyrosine and histidine residues in the collagen. Tyrosine (TyrOH) reacts with hydrogen peroxide formed by riboflavin and produces phenoxyl radicals (TyrO·) and bityrosine by radical-radical coupling (Pichorner and others 1995).

\[
\begin{align*}
2 \text{TyrOH} + \text{H}_2\text{O}_2 & \rightarrow 2 \text{TyrO}^\cdot + 2\text{H}_2\text{O} \\
\text{TyrO}^\cdot + \text{TyrO}^\cdot & \rightarrow \text{TyrO}-\text{OTyr (bityrosine)}
\end{align*}
\]
2.2.5.2 Enzymes

The photosensitized modification of proteins by riboflavin often inactivates enzymes. Reactive oxygen species formed by photosensitized riboflavin cause crosslinks in enzymes, protein denaturation, and finally a loss of enzymatic activity. Singlet oxygen quenchers such as α-tocopherol acetate, β-carotene, sodium azide, and ascorbic acid can prevent protein crosslinking and photoinactivation of enzymes (Dalle Carbonare and Pathak 1992). Catalase and lysozyme are inactivated by Type-I and Type-II mechanisms (Edwards and Silva 2001). Among the 20 amino acids present in lysozyme, only tryptophan, tyrosine, and histidine are modified by riboflavin-induced photochemical damage (Edwards and Silva 2001). Visible light irradiation on horseradish peroxidase in the presence of riboflavin decreases the enzyme activity only when the enzyme is non-glycosilated.

2.2.5.3 Lipids

Riboflavin can photosensitize the cis-trans isomerization of olefinic compounds. Lipid oxidation sensitized by riboflavin is another important factor in the development of light-induced flavor, although the action of riboflavin on lipid degradation is lower than on protein (Aurand and others 1966; Dimick 1982). Unsaturated fatty acids or esters are efficiently photooxidized in the presence of the riboflavin derivative, riboflavin-2', 3', 4', 5'-tetraacetate, by the Type-II mechanism (Fukuzumi and others 1989). Polyunsaturated fatty acids such as linoleic acid in foods or milk products can be easily oxidized under
light by singlet oxygen (Figure 15). The reaction rate of singlet oxygen with linoleic acid is approximately 1450 times faster than triplet oxygen free radical oxidation (Rawls and VanSanten 1970).

![Figure 15 Singlet oxygen oxidation of linoleic acid.](image)

As vegetable oils do not contain riboflavin, photosensitized oxidation of vegetable oils by riboflavin is not problematic. Oxygen uptake by fish oil under light is higher in
the presence of riboflavin than in the absence of riboflavin. Riboflavin has no effect on the oxygen uptake of fish oil stored in the dark. Fish oil is oxidized in the presence of riboflavin under light via the Type-II mechanism (Davis and others 1995).

Riboflavin and lumiflavin can undergo a facile photooxygenation of cholesterol derivatives in acetonitrile and pyridine, producing 6-en-5α'-hydroperoxide and 5-en-7-hydroperoxide. The singlet oxygen formed by riboflavin or lumiflavin reacts with the double bond of cholesterol and the adjacent double-bond migrates, as shown in Figure 16 (Wu and others 2001).

![Figure 16](image)

Figure 16 Cholesterol oxidation by photosensitized riboflavin.

The yields of 6-en-5α’-hydroperoxide and 5-en-7-hydroperoxide vary depending on the experimental conditions (Wu and others 2001). More 6-en-5α’-hydroperoxide (90%) is produced when cholesterol in acetonitrile is reacted in the presence of riboflavin.
and oxygen under light. However, when lumiflavin is used as a photosensitizer, cholesterol in pyridine and acetonitrile produces more 6-en-5a’- hydroperoxide (86%) and 5-en-7- hydroperoxide (80%), respectively.

2.2.5.4 Carbohydrates

Carbohydrates are relatively insensitive to photooxidation as compared to lipids and proteins (Min and Boff 2002). Riboflavin stimulates the oxidation of glucose under visible light via reactive oxygen species such as singlet oxygen, superoxide anion, hydrogen peroxide, and hydroxyl radical (Goodman and Hochstein 1981). The light-dependency of glucose oxidation is not affected by catalase or superoxide dismutase, but is inhibited by a singlet oxygen scavenger (Goodman and Hochstein 1981). Photooxidation of glucose depends on the riboflavin concentration, pH of the reaction medium, and the glucose concentration (Silva and others 1999). At riboflavin concentrations of $10^{-6}$ M, glucose oxidation is stimulated by approximately 30%. When the riboflavin concentration is higher than $10^{-5}$ M, light enhances the oxidation of glucose by 2 to 3 fold. A higher pH also favors the glucose photooxidation by riboflavin under oxygen. The oxidation rate of glucose photosensitized by riboflavin at a pH of 4.0, 7.0, and 10.0 were $3.79 \times 10^{-3}$, $8.30 \times 10^{-3}$, and $5.50 \times 10^{-2}$ mM oxygen/min, respectively. Riboflavin acts preferentially as a Type-I sensitizer at a high glucose concentration of 20% (Silva and others 1999).
2.2.5.5 Vitamins

The riboflavin photosensitized oxidation causes destruction of vitamin A, vitamin C, vitamin D, and vitamin E. Sunlight destroys Vitamin A and its esters in milk by ring opening (DeMan 1981). Vitamin A loss increases with the length and intensity of light exposure, but decreases with the fat content of milk (Whited and others 2002). The oxidation products of vitamin A in the presence of riboflavin and oxygen under light are ethyl-(2, 6, 6-trimethylcyclohex-1-ene) carboxylate, retinal, 5, 8-peroxide of β-ionone, 5, 6-peroxide of vitamin A, and retinoic acid, as shown in Figure 17. Photooxidation of the palmitate ester of vitamin A sensitized by riboflavin causes cleavage of the side chain double bonds and produces aldehyde compounds.

Ascorbic acid is an excellent antioxidant and it does not absorb visible light, but it is rapidly photooxidized with a high consumption of oxygen in the presence of riboflavin. The photooxidation of ascorbic acid by riboflavin proceeds essentially via the Type-I mechanism and the oxidation products of ascorbic acid contain carbonyl groups (de La Rochette and others 2000). Photodecomposition of ascorbic acid by riboflavin under oxygen also involves singlet oxygen (Sahbaz and Somer 1993; Jung and others 1995). The reaction rate of singlet oxygen with ascorbic acid is higher than that with α-tocopherol (Jung and others 1991), but is lower than that with β–carotene (Jung and Min 1991). The photosensitizing activity of riboflavin on ascorbic acid oxidation is higher than that of methylene blue, rose Bengal, or protoporphyrin IX. The relative photosensitizing activity of riboflavin, methylene blue, and protoporphyrin IX in photooxidation of ascorbic acid was reported as 21:15:1 (Jung and others 1995).
Figure 17  Vitamin A oxidation by photosensitized riboflavin.
The photodegradation of ascorbic acid by riboflavin sensitization under oxygen depends on many factors. These include light intensity, concentrations of riboflavin, oxygen, and ascorbic acid, the temperature and pH of the reaction media, and presence of other components (Sahbaz and Somer 1993; Jung and others 1995; Sansal and Somer 1997; de La Rochette and others 2000). The riboflavin sensitized oxidation of ascorbic acid increases with the light intensity and concentration of riboflavin (Jung and others 1995). As the riboflavin concentration in 0.01 M potassium phosphate buffer (pH 7.5) increases from 0 to 1.2, 2.4, 3.6, and 6.0 ppm, the percent of ascorbic acid lost after 6 minutes of illumination is 2.1, 35.4, 58.3, 74.0, and 89.6%, respectively. In an anaerobic environment, ascorbic acid photooxidation is negligible; however, it sharply increases with rising oxygen pressure and was maximal at 100% oxygen (de La Rochette and others 2000). Decomposition of ascorbic acid in the presence of riboflavin under light increases with the ascorbic acid (H\textsubscript{2}A) concentration, (especially the concentration of ascorbate ion) and temperature (Sansal and Somer 1997). The singlet oxygen oxidation of ascorbic acid occurs slowly at lower pH. The rate constant for the reaction of ascorbic acid with singlet oxygen at pH 7.5, 6.0, and 4.5 are 6.63 x 10\textsuperscript{8}, 5.77x 10\textsuperscript{8}, and 5.27x 10\textsuperscript{8} M\textsuperscript{-1}s\textsuperscript{-1}, respectively (Jung and others 1995). The reaction rate for the ascorbic acid with singlet oxygen is 3.08 x 10\textsuperscript{8} M\textsuperscript{-1}s\textsuperscript{-1} in an aqueous solution of pH 7 and 25\textdegree C (Yang and Min 1994).

Sansal and Somer (1997) proposed that riboflavin associates with ascorbic acid. Under anaerobic conditions, ascorbic acid (H\textsubscript{2}A) that has been ionized to ascorbate ion (HA\textsuperscript{-}) in a citrate buffer at pH 4.5 is decomposed via a radical pathway in the presence of riboflavin and light (Sansal and Somer 1997) as shown in Figure 18. The complex of
riboflavin and ascorbate can produce riboflavin radical and an ascorbic acid radical upon irradiation. Dehydroascorbic acid (A) can be produced by two molecules of the ascorbic acid radical or by the direct reaction between an excited triplet riboflavin and ascorbic acid.

![Chemical Reaction Diagram]

Figure 18 Decomposition of ascorbic acid.

Riboflavin accelerates the oxidation of vitamin D by light via the singlet oxygen pathway (Li and others 2000, Li and Min 1998). The major oxidation product is 5, 6-epoxide of vitamin D (King and Min 2002). Riboflavin has no effect on vitamin D oxidation in the dark (King and Min 1998). The reaction rate constant of vitamin D with singlet oxygen produced by riboflavin is $2.2 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$ (Li and others 2000). Vitamin D degradation caused by singlet oxygen is independent of temperature. However,
degradation of vitamin D caused by triplet oxygen under light and in the dark is temperature dependent (Li and others 2000).

2.2.6 Effects of riboflavin on the sunlight flavor of milk.

Sunlight flavor formation in milk has been recognized and extensively studied. The sunlight flavor is generally described as sulfury. Methionine has been purported to be involved in the formation of sunlight flavor of milk in the presence of riboflavin (Patton and Josephson 1953). Methional and dimethyl sulfide were the compounds assumed to be responsible for the sunlight flavor. The mechanisms for the formation of sunlight flavor have been very controversial. Singlet oxygen is formed from molecular triplet oxygen in the presence of riboflavin in milk under sunlight. Jung and others (1998) reported that the singlet oxygen reacts with the electron-rich sulfur in methionine to form hydroperoxide. The hydroperoxide on sulfur decomposes to methional and thiomethyl radicals, as shown in Figure 19. The reaction between thiomethyl radicals produces dimethyl disulfide. The dimethyl disulfide concentration correlates well with the sunlight flavor sensory score (Jung and others 1998). The proportion of methional increases under the nonaqueous solvent (Foote 1979). The addition of ascorbic acid reduces dimethyl disulfide formation in milk and improves the sensory quality of milk (Jung and others 1998).
2.2.7 Conclusions

Riboflavin is relatively stable during food processing and storage, except under light. Riboflavin absorbs light energy to produce an excited triplet state riboflavin. Triplet riboflavin undergoes Type-I and Type-II photosensitization pathway. Radicals of
riboflavin, food components, and reactive oxygen species such as superoxide anion radicals, singlet oxygen, hydroxyl radical, and hydrogen peroxide, are produced from the Type-I and Type-II mechanisms, respectively. Radicals and reactive oxygen species accelerate the oxidation of food components and cause nutrient loss. Decomposition of the nutrients depends on light exposure, concentration of oxygen and riboflavin, and the presence of other components. Protein degradation sensitized by riboflavin under light occurs primarily by the Type-I pathway at low oxygen concentrations, while the Type-II pathway is favored at high oxygen concentrations. The reaction of singlet oxygen formed by riboflavin and methionine under light produces dimethyl disulfide, which is responsible for the sunlight flavor of milk. Singlet oxygen is involved in lipid and cholesterol oxidation in the presence of riboflavin under light. Carbohydrates are less sensitive to riboflavin photosensitized oxidation than proteins or lipids. At high glucose concentrations, the Type-I pathway is favored. Vitamins A, C, and D are decomposed by the Type-I, or by singlet oxygen pathway (Type-II) in the presence of riboflavin under light. Riboflavin is an excellent photosensitizer for singlet oxygen formation. It is also a super reactant for singlet oxygen with the reaction rate of $1.01 \times 10^{10} \text{ M}^{-1}\text{s}^{-1}$. 

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3.1 Abstract

The formation of singlet oxygen by riboflavin and the kinetics and mechanisms of riboflavin degradation in aqueous solution under light were determined. The singlet oxygen formation rate by riboflavin was 2.31 µmole oxygen/mL headspace.h of the serum bottle. The degradations of riboflavin were 66% in D$_2$O and 40% in H$_2$O, respectively, under light after 24 h. The results indicated that singlet oxygen was involved in riboflavin destruction under light. The riboflavin destructions were 94.0% and 15.7% with 0 mM or 160 mM ascorbic acid, respectively, under light after 96 h. The reaction rate between riboflavin and singlet oxygen was 1.01 x 10$^{10}$ M$^{-1}$s$^{-1}$, which is a diffusion-controlled reaction rate. This explained the rapid degradation of riboflavin in foods under light. Ascorbic acid and sodium azide reduced the degradation of riboflavin
under light with different quenching mechanisms. Ascorbic acid quenched both singlet oxygen and excited triplet riboflavin. Sodium azide quenched only the singlet oxygen in riboflavin solution with a quenching rate of $1.547 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$. Considering the involvement of both the Type-I and the Type-II mechanisms in riboflavin degradation under light, singlet oxygen quencher alone could not protect the riboflavin from degradation completely. Addition of ascorbic acid can protect riboflavin oxidation in foods exposed to light.

3.2 Introduction

Riboflavin is naturally present in milk, eggs, meat products, and vegetables (Szczesniak and others 1971; Gliszczynska and Koziolowa 1999; USDA 2003). It is also used to fortify processed foods (Singh and others 1996; Chen and others 2001). It is an essential nutrient because the human body has to obtain this compound from the diet. Riboflavin is an active part in the coenzymes of flavin mononucleotide (FMN) and flavin-adenine dinucleotide (FAD). These coenzymes play essential roles in the activities of several dehydrogenases and oxidases. Riboflavin has a complex photochemistry because of its ability to accept or lose a pair of hydrogen atoms (Isaka 1955; Kim and others 1993; Crank and Pardijanto 1995; Li and Min 1998; Edwards and others 1999; Viteri and others 2003; Criado and others 2003). Riboflavin under light can generate reactive oxygen species such as superoxide anions and singlet oxygen (Kumari and others 1996; Grzelak and others 2001; Min and Boff 2002; Mahns and others 2003). Formation
of reactive oxygen species by riboflavin under light proceeds by both the Type-I and the Type-II mechanisms (de La Rochette and others 2003). After receiving energy from light, riboflavin becomes the excited triplet riboflavin. In the Type-I mechanism, the excited triplet riboflavin is reduced by abstraction of electrons or hydrogen ions from other food components to form riboflavin radical. In the Type-II mechanism, the excited triplet riboflavin reacts with atmosphere triplet oxygen to produce superoxide anion or singlet oxygen (Foote 1985). 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 (Kumari and others 1996; Min and Boff 2002; Mahns and others 2003).

Reactive oxygen species can cause not only the nutrient’s destruction, but also the off-flavor formation in foods. While it is relatively stable during food processing and storage in the dark, riboflavin is very sensitive to UV and visible light. Thirty percent of the riboflavin in milk was lost after exposure to sunlight for 30 min (Wishner 1964). Twenty to thirty percent of the riboflavin was degraded under light with wavelength between 290 and 400 nm (Joshi 1989). It was reported that the decomposition products from riboflavin in milk under light were lumiflavin and lumichrome (Toyosaki and Hayashi 1993). It took only a few minutes of the simulated sunlight exposure to degrade riboflavin to lumichrome (Tatsumi and others 1992).

Singlet oxygen may be involved in riboflavin degradation under light (Lee and others 1998). Although many studies on the photosensitization and degradation of riboflavin have been published, the detailed chemical mechanisms and kinetics have not been reported. The objectives of this research are (1) to prove the involvement of single
oxygen in riboflavin degradation under light, (2) to determine the reaction rate between singlet oxygen and riboflavin, and (3) to study the effects of ascorbic acid and sodium azide on the riboflavin degradation under light and their reaction mechanisms.

3.3 Materials and Methods

Materials

Riboflavin and ascorbic acid were purchased from Acros Organics (Jersey City, NJ). Deuterium oxide and sodium azide were purchased from Sigma Chemical Co. (St. Louis, MO). Water used in all sample preparations was purified by a Milli-Q purification system (Millipore Co., Bedford, MA). Serum bottles (10 and 35 mL), aluminum caps, and Teflon™-coated septa were purchased from Supelco, Inc. (Bellefonte, PA).

Sample preparations

A 50 µM riboflavin solution in H₂O or D₂O was prepared to study the effect of light on riboflavin degradation. A 2 mL aliquot of 50 µM riboflavin solution was pipetted into a 10 mL serum bottle and sealed to be air-tight with a Teflon™-coated rubber septum and an aluminum crimp cap. The bottles were stored in duplicate in a light box of 1000 lux (Li and others 2000) or in the dark at 30°C. Riboflavin concentration in the serum bottles was determined in duplicate after 2.5, 4.5, 6.5, 20.5, and 24.5 h of storage in the light box.
Dairy cream with 36% milk fat was diluted with deionized water to obtain 0.10, 0.14, 0.21, and 0.41 M milk fat to study the singlet oxygen formation by 40 µM riboflavin under light. The 30 mL sample was pipetted into a 35 mL serum bottle and sealed to be air-tight with a Teflon™-coated rubber septum and an aluminum cap. Sample bottles were then placed in a light box for 3 h. Oxygen depletion in the headspace of the sample bottles was analyzed by gas chromatography with a thermal conductivity detector to determine the singlet oxygen formation rate in the headspace of the sample bottles (King and Min 1998).

Ascorbic acid solutions of 0, 10, 20, 40, 80, and 160 mM in riboflavin concentrations of 12.5, 25, 50, or 100 µM were prepared to study the effect of ascorbic acid on the riboflavin degradation under light. Samples in serum bottles were stored in the light box of 1000 lux at 30°C for up to 96 h (Li and others 2000). Riboflavin concentration in the samples was determined in duplicate after the bottles were stored in the light box for 0, 2, 4, 6, 24, 48, 72, and 96 h.

Sodium azide concentrations of 0, 0.44, 3.3, 6.7, and 10.0 mM were added to a riboflavin solution of 100 µM to study the effect of sodium azide on riboflavin degradation under light. Riboflavin concentration in the samples was determined in duplicate after the bottles were stored in the light box for 0, 24, 48, and 72 h.

Riboflavin solutions of 12.5, 25, 50, and 100 µM in H₂O containing ascorbic acid of 0, 10, 20, 40, 80, and 160 mM were prepared to determine the reaction rate between singlet oxygen and riboflavin and the total quenching rate of ascorbic acid for singlet oxygen. Riboflavin solutions of 12.5, 25, 50, and 100 µM in H₂O containing sodium azide of 0, 1, 5, 10, and 20 mM were also prepared to determine the reaction rate
between singlet oxygen and riboflavin and the total quenching rate of sodium azide for singlet oxygen. A 2 mL aliquot of the prepared solution was placed in a 10 mL serum bottle and stored in the light box as described above for 24 h. A steady state kinetic equation was applied (Li and others 2000).

Riboflavin analysis

Riboflavin in aqueous solution was monitored by measuring absorbance at 447 nm with a UV-Vis spectrophotometer (Shimadzu, Tokyo, Japan). The concentration of riboflavin was calculated using the molar extinction coefficient of 12500 M$^{-1}$cm$^{-1}$ (Edwards and others 1999). Riboflavin concentration of the samples was also determined by HPLC analysis (HP 1100; Hewlett-Packard, Wilmington, DE) equipped with an Aqua C-18 column (5 µ, 150 mm x 4.60 mm) (Phenomenex, Torrance, CA). Samples were filtered with a 0.2 µm membrane filter. The injection volume was 10 µL. The flow rate of the mobile phase (methanol: 0.05 M ammonium acetate=3:7 (v/v)) was 1.0 mL/min. The absorbance of the separated riboflavin was measured at 447 nm with a HP 1100 Diode Array Detector. Riboflavin concentration was calculated using a standard linear line of HPLC peaks of different concentrations of riboflavin in aqueous solutions.

Headspace oxygen analysis

Headspace oxygen in the sample bottles was analyzed by injecting a 100 µL headspace sample into a HP 5890 GC equipped with a stainless steel molecular sieve column (13X, 80:100; Alltech, Deerfield, IL) and a thermal conductivity detector (King
and Min 1998). High-purity helium (99.99%) was used as the carrier gas. The flow rate was 40 mL/min. The GC oven temperature was maintained at 40°C. The temperatures at the injector port and detector were maintained at 120 and 150°C, respectively. Duplicated injections were performed for each sample bottle. Electronic counts were integrated on a HP 3396A integrator (King and Min 2002; Yang and others 2002). The depleted headspace oxygen content was expressed as µmoles of oxygen per mL of headspace gas (Bradley 1991). One mL of air contains 9.35 µmoles of oxygen. The gas chromatographic peak area of 9.35 µmoles of oxygen was measured in electronic counts by injecting 100 µL of air into the gas chromatograph. Electronic counts of 1 µmole oxygen were calculated (Bradley 1991).

Statistical analysis

All the experimental treatments were done in duplicate. ANOVA or T-test was used to analyze the data.

3.4 Results and Discussion

Reproducibility of spectrophotometric and HPLC analyses for riboflavin

Riboflavin in aqueous solution was quantitated spectrophotometrically at 447 nm. The coefficient of variations of the spectrophotometric method for 6 replicated analyses was 2.5% (data not shown). Riboflavin concentration in the samples was also determined
by HPLC. The concentration was determined by comparing to the standard linear line of different concentrations of authentic riboflavin. The coefficient of variations of HPLC analysis for 6 replicated samples was 2.7%. The difference between the spectrophotometric method and the HPLC method for riboflavin determination is that the spectrophotometric method did not separate riboflavin from the sample, while the HPLC method did separate the riboflavin from the sample and measured the riboflavin at 447 nm. The results from the spectrophotometric and HPLC methods were not significantly different at $\alpha=0.05$. The data also indicated that the absorbance at 447 nm in the spectrophotometric determination was due to riboflavin only.

Effect of light on riboflavin degradation in aqueous solution

The changes of 50 µM riboflavin in samples under light or in dark at 30°C are shown in Table 2. Riboflavin concentration decreased as storage time increased. The degradation of riboflavin was faster under light than in the dark. Riboflavin degradation was 3.6% in dark and 98% under light after 96 h. Light accelerated the degradation of riboflavin, which agrees with the findings by others (Wishner 1964; Furuya and others 1984; Lee and others 1998). Figure 20 shows the retention of riboflavin in H$_2$O or D$_2$O during storage under light or in the dark. Under light, the riboflavin degradation in D$_2$O was higher than that in H$_2$O. However, the riboflavin degradations in D$_2$O and in H$_2$O in the dark were the same (data not shown). This result suggested the involvement of singlet oxygen in the riboflavin degradation in aqueous solution under light. Riboflavin has been reported to produce singlet oxygen from triplet oxygen under light. The lifetime
of singlet oxygen in D$_2$O is about 13 times longer than that in H$_2$O (Gorman and Rogers 1989; Li 1997). The longer lifetime of singlet oxygen in D$_2$O increased the reaction between singlet oxygen and riboflavin, resulting in higher riboflavin degradation.

Singlet oxygen formation rate in aqueous media containing 40 µM riboflavin

The headspace oxygen depletion in the sample bottle with 40 µM riboflavin increased with the concentration of milk fat. As the milk fat concentration increased from 0.10, 0.14, 0.21, to 0.41 M, the depleted headspace oxygen increased from 1.79, 2.32, 2.90, to 4.10 µmole of oxygen per mL of headspace. Figure 21 illustrates the reciprocal plotting of the depleted oxygen in headspace against the milk fat concentration, which can be used to determine the singlet oxygen formation rate (King and Min 1998). The intercept of the reciprocal plotting is the inverse of the singlet oxygen formation rate, which was 0.1441 mL headspace/µmole singlet oxygen (Figure 21). The inverse of this intercept is 6.94 µmole oxygen/mL headspace in 3 h. Therefore, the rate of singlet oxygen formation by 40 µM riboflavin in the milk fat aqueous emulsion system was 2.31 µmole oxygen/mL.headspace.h. King and Min (1998) reported that the singlet oxygen formation rate by 15 ppm riboflavin in 12% water/88% acetone was 1.57 µmol oxygen/mL.headspace·h in a serum bottle.

Effect of ascorbic acid on riboflavin degradation under light

The effect of ascorbic acid on riboflavin degradation in aqueous solution under light is summarized in Table 3. Without the addition of ascorbic acid, riboflavin
degraded very quickly in all of the riboflavin concentrations studied. At starting riboflavin concentrations of 50 µM and 100 µM, over 90% of the riboflavin in the solution was degraded after storing under light for 72 and 96 h. At lower starting riboflavin concentrations, the degradation quickened. It took less than 24 h to degrade >90% of the riboflavin in solution with starting concentrations of 12.5 µM and 25 µM.

Riboflavin acted as both a photosensitizer and as a reactant in the photosensitized degradation system. In aqueous solution, the O₂ solubility is lower than in other non-polar solvent systems. The lower O₂ availability would favor the photosensitized reaction toward the Type-I mechanism. If the starting riboflavin concentration is high, then the Type-I mechanism would be even more favored because it provided a higher reactant concentration for the Type-I reactions.

Increasing the concentration of ascorbic acid in the riboflavin solution significantly reduced riboflavin degradation. As shown in Table 3, when 40 mM of ascorbic acid was included in the 100 µM riboflavin solution, 60.5% of the riboflavin remained in the solution after 96 h of light exposure. Under the same condition, only 6% of riboflavin remained in the solution when ascorbic acid was not included in the sample preparation. This clearly indicated that ascorbic acid protected riboflavin from degradation under light.

Effect of sodium azide on riboflavin degradation under light

Riboflavin degradation in aqueous solutions containing 100 µM riboflavin and 0, 0.44, 3.3, 6.7, and 10 mM sodium azide are shown in Figure 22. Sodium azide
minimized the riboflavin degradation under light. As one of the most effective singlet oxygen quenchers, sodium azide has been used in numerous investigations to understand if singlet oxygen plays significant roles in particular oxidation systems (Kanofsky 1991; Fujita and Matsuo 1994). Our data proved the involvement of singlet oxygen in riboflavin degradation under light.

Because sodium azide only quenches singlet oxygen, the effect of sodium azide on the degradation of riboflavin can be used to estimate the relative contributions of the Type-I or Type-II mechanisms on the degradation of riboflavin under light. The riboflavin degradation without any singlet oxygen quencher under light is due to the sum of both the Type-I and Type-II mechanisms. The degradation observed at higher concentrations of singlet oxygen quencher are mainly attributed to the Type-I mechanism. As shown in Figure 22, the riboflavin degradation decreased with the addition of sodium azide. More than 75% of the photosensitized riboflavin degradation can be prevented by the addition of 10 mM sodium azide. This data implicates the Type-I mechanism as being responsible for 25% of the total photosensitized riboflavin degradation at 100 µM initial concentration.

Reaction rate between riboflavin and singlet oxygen, and mechanisms for riboflavin degradation reduction by ascorbic acid and sodium azide under light

Riboflavin is a water-soluble photosensitizer for singlet oxygen formation (Bradley and others 2003) and also an excellent reactant for singlet oxygen (Min and
The schematic diagram for the formation of oxidized riboflavin (RFO$_2$) via singlet oxygen oxidation, which is formed by riboflavin under light, is shown below:

When riboflavin (RF) absorbs energy from light, it becomes an excited singlet riboflavin ($^1$RF*). Through an intersystem crossing mechanism, the excited riboflavin becomes an excited triplet riboflavin ($^3$RF*). Excited triplet riboflavin may interact with a quencher (Q) to become a ground-state riboflavin or may interact with triplet oxygen ($^3$O$_2$) to produce singlet oxygen ($^1$O$_2$). Singlet oxygen may naturally decay, may react with singlet riboflavin to form the oxidized riboflavin (RFO$_2$), or may be quenched physically or chemically by quenchers (Min and Boff 2002).

The steady-state kinetic equation for singlet oxygen and the excited riboflavin quenching in the presence of riboflavin as a photosensitizer and as a reactant for singlet oxygen oxidation is as follows (Foote 1979; Yang and others 2002):

$$\frac{d[RFO_2]}{dt} = K^{-1} \left(1 + \frac{k_o [O]}{k_q [^3O_2]} \right) \left(1 + \frac{(k_q + k_{ox-Q})[Q]}{k_r} \right) \times [RF]^{-1}$$

where [RFO$_2$] is the concentration of oxidized riboflavin; K is the quantum yield of intersystem crossing; $k_Q$ is the reaction constant for triplet riboflavin quenching; [Q] is the quencher concentration; $k_o$ is the reaction constant for singlet formation by energy transfer from excited triplet riboflavin to triplet oxygen; $[^3O_2]$ is the triplet oxygen
concentration; $k_q$ is the reaction constant of singlet oxygen physical quenching; $k_{\text{ox-Q}}$ is the reaction constant of singlet oxygen chemical quenching; $k_d$ is the reaction rate of singlet oxygen decay in a solvent; $k_r$ is the reaction constant between riboflavin and singlet oxygen; and $[RF]$ is the initial concentration of riboflavin.

When there is only the singlet oxygen quenching involved, the equation can be simplified as follows:

$$\left\{ \frac{d[RFO_2]}{dt} \right\}^{-1} = K^{-1} \left( 1 + \frac{(k_q + k_{\text{ox-Q}})[Q] + k_d}{k_r} \times [RF]^{-1} \right)$$

Plots of $[RFO_2]^{-1}$ against $[RF]^{-1}$ at various concentrations of quencher results in similar y-intercepts and different slopes. The intercept of this plot would be equal to $K^{-1}$ and the slope would be equal to $K^{-1} \times \frac{(k_q + k_{\text{ox-Q}})[Q] + k_d}{k_r}$. The ratio of the slope to intercept (S/I) would become $\frac{(k_q + k_{\text{ox-Q}})[Q] + k_d}{k_r}$, which is independent of $O_2$ concentration. A new graph S/I plotted against $[Q]$ would result in a line with an intercept of $\frac{k_d}{k_r}$ and a slope of $\frac{k_q + k_{\text{ox-Q}}}{k_r}$.

If there is only excited triplet riboflavin quenching, then the equation becomes:

$$\left\{ \frac{d[RFO_2]}{dt} \right\}^{-1} = K^{-1} \left( 1 + \frac{k_q[Q]}{k_o[O_2]} \right) \left( 1 + \frac{k_d}{k_r} \times [RF]^{-1} \right)$$

Both the y-intercept and the slope in the plot of $[RFO_2]^{-1}$ against $[RF]^{-1}$ vary according to the concentration of a quencher acting on the excited triplet riboflavin. The y-intercept is equal to $K^{-1} \left( \frac{k_o[O_2] + k_q[Q]}{k_o[O_2]} \right)$ and the slope is equal to...
to \(K^{-1}\{\frac{k_d(k_o[3O_2^+] + k_Q[Q])}{k_o[3O_2^+]}\}\). The ratio of the slope to intercepts, \(\frac{k_d}{k_r}\), is the same regardless of the quencher concentration.

When there are both triplet riboflavin quenching and singlet oxygen quenching, the y-intercept is equal to \(K^{-1}\{\frac{k_o[3O_2^+] + k_Q[Q]}{k_o[3O_2^+]}\}\) and the slope is equal to \(K^{-1}\{\frac{(k_o[3O_2^+] + k_Q[Q])((k_q + k_{ao-\sigma})([Q] + k_d))}{k_o[3O_2^+]}\}\). The ratio of the slope to y-intercept is \(\frac{(k_q + k_{ao-\sigma})([Q] + k_d)}{k_r}\). All of these terms vary with the quencher concentrations.

When there is no quencher added in an experimental system, the equation is further simplified to the following (Foote 1979; Jung and others 1995; Lee and others 1997):

\[
\{\frac{d[RFO_2]}{dt}\}^{-1} = K^{-1}(1 + \frac{k_d}{k_r}[RF]^{-1})
\]

When plotting \([RFO_2]^{-1}\) against \([RF]^{-1}\), the ratio of the slope to intercept of the plot is \(\frac{k_d}{k_r}\). If the singlet oxygen decay rate \(k_d\) is known, it is possible to determine the reaction rate constant, \(k_r\), between riboflavin and singlet oxygen (Foote 1979). The singlet oxygen decay rate (\(k_d\)) in water has been reported as \(2.56 \times 10^5\) s\(^{-1}\) (Rodgers and Snowden 1982; Hurst and Schuster 1983).

The reciprocal plot of degraded riboflavin concentration against the initial riboflavin concentrations of 12.5, 25, 50, and 100 µM in H\(_2\)O under light for 24 h is shown in Figure 23. The linear regression equation of \([RFO_2]^{-1}\) plotted against \([RF]^{-1}\) is
Y=0.6214X + 24.513 (R²=0.99), where X and Y are the reciprocals of initial riboflavin concentrations and the oxidized riboflavin concentrations, respectively. The ratio of the slope to intercept of this regression line is equal to \( k_d/k_r \), which is \( 2.53 \times 10^{-2} \) mM. Considering the \( k_d \) in water is \( 2.56 \times 10^5 \) s\(^{-1}\) (Rodgers and Snowden 1982; Hurst and Schuster 1983), the reaction rate \( (k_r) \) between riboflavin and singlet oxygen is \( 2.56 \times 10^5 \) s\(^{-1}\)/\( 2.53 \times 10^{-2} \) mM = \( 1.01 \times 10^7 \) M\(^{-1}\)s\(^{-1}\) = \( 1.01 \times 10^{10} \) M\(^{-1}\)s\(^{-1}\).

The reciprocal plots of degraded riboflavin concentration against the initial riboflavin concentration in the presence of various concentrations of ascorbic acid under light for 24 h are shown in Figure 23. As the ascorbic acid concentration increased, the oxidized riboflavin concentration decreased. Both the slope and the y-intercept of the reciprocal plot increased as the ascorbic acid concentration increased. Different slopes, different y-intercepts, and different ratios of the slope to y-intercepts (Table 4) indicated that ascorbic acid quenched both singlet oxygen and excited triplet riboflavin under light. Since the reaction constant \( (k_o) \) for singlet formation by energy transfer from excited triplet riboflavin to triplet oxygen is unknown, the quenching rate of ascorbic acid on the excited triplet riboflavin can not be obtained.

The effects of 0, 1, 5, 10, and 20 mM sodium azide on riboflavin degradation at various starting riboflavin concentrations after 24 h under light are shown in Figure 24. As the sodium azide concentration increased from 0 to 20 mM, the riboflavin degradation decreased. The slopes of the reciprocal plot in Figure 24 increased significantly with sodium azide concentration. However, the y-intercepts of the plots were not significantly different (P>0.05). This clearly indicates that sodium azide quenched only singlet oxygen (Foote 1979).
The plot of slope/intercept against various sodium azide concentrations is shown in Figure 25. A regression line $Y=0.002447 X +0.0405$ ($R^2=0.85$) is obtained. The intercept of this regression line will give $\frac{k_d}{k_r}$ and the slope of this line yields $\frac{k_q + k_{\text{ox-Q}}}{k_r}$ (King and Min 1998; Yang and others 2002). Using the singlet oxygen decay rate in water, $k_d = 2.56 \times 10^5 \text{s}^{-1}$ (Hurst and Schuster 1983), $k_r$ can be calculated as $2.56 \times 10^5 \text{s}^{-1}/0.0405 \text{mM}=6.32 \times 10^6 \text{mM}^{-1}\text{s}^{-1}=6.32 \times 10^9 \text{M}^{-1}\text{s}^{-1}$. The slope 0.002447 equals $\frac{k_q + k_{\text{ox-Q}}}{k_r}$, therefore, the total singlet oxygen quenching rate ($k_q + k_{\text{ox-Q}}$) of sodium azide is $6.32 \times 10^9 \text{M}^{-1}\text{s}^{-1} \times 0.002447 = 1.547 \times 10^7 \text{M}^{-1}\text{s}^{-1}$.

The rate constants obtained experimentally may be affected by a number of factors such as the temperature (Li and others 2001), the pH (Mashiko and others 1991), the ionic strength of solvent systems (Rubio and others 1992), and the ratio of organic solvents in the aqueous mixture (Li and others 2001). The types of sensitizers for singlet oxygen generation may also affect the quenching rate constant (Hall and Chignell 1987). The singlet oxygen quenching rate of sodium azide is consistent with previous findings (Haag and Mill 1987).

3.5 Conclusions

Riboflavin produces singlet oxygen from triplet oxygen under light. The reaction rate between riboflavin and singlet oxygen is $1.01 \times 10^{10} \text{M}^{-1}\text{s}^{-1}$. This reaction rate is one of the fastest known chemical reaction rates. The exceptionally high chemical reaction
rate clearly explains why the riboflavin is so easily destroyed under light. The electrophilic singlet oxygen reacts with riboflavin to destroy the riboflavin. Riboflavin is a good sensitizer for singlet oxygen formation under light and is also a good reactant for singlet oxygen. Ascorbic acid or sodium azide can protect riboflavin under light. However, the quenching mechanisms are different between ascorbic acid and sodium azide. Ascorbic acid quenched both the singlet oxygen and the excited triplet riboflavin under light, while sodium azide only quenched the singlet oxygen. The singlet oxygen quenching rate of sodium azide was $1.547 \times 10^7 \text{ M}^{-1}\text{s}^{-1}$. Riboflavin degradation under light involves both Type-I and Type-II mechanisms. Singlet oxygen quencher alone can not completely protect the riboflavin from degradation under light. Ascorbic acid, which is a quencher of both the singlet oxygen and the excited triplet riboflavin, can effectively protect riboflavin in foods under light.

3.6 References


<table>
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<tr>
<th>Storage time (h)</th>
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<th>24</th>
<th>48</th>
<th>72</th>
<th>96</th>
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<td>49.2</td>
<td>21.6</td>
<td>9.4</td>
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<tr>
<td>In the dark</td>
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<td>99.8</td>
<td>99.4</td>
<td>98.2</td>
<td>96.4</td>
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Table 2  Effects of light on the retention (%) of riboflavin (50 µM) at 30 °C.
<table>
<thead>
<tr>
<th>Riboflavin (µM)</th>
<th>Ascorbic acid (mM)</th>
<th>Riboflavin retention (%) during storage (h)</th>
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<tr>
<td>12.5</td>
<td>0</td>
<td>100 92.8 82.4 n.d. 4.0 0.8 n.d. n.d.</td>
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<td>10</td>
<td>100 94.4 86.4 n.d. 48.8 24.8 n.d. n.d.</td>
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<td>100 96.0 89.6 n.d. 58.4 35.2 n.d. n.d.</td>
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<td>40</td>
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<tr>
<td></td>
<td>80</td>
<td>100 97.6 94.4 n.d. 79.2 64.8 n.d. n.d.</td>
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<td></td>
<td>160</td>
<td>100 98.4 96.8 n.d. 88.0 76.8 n.d. n.d.</td>
</tr>
<tr>
<td>25</td>
<td>0</td>
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<td>10</td>
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</tr>
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<td></td>
<td>160</td>
<td>100 100 99.7 99.4 96.1 91.6 88.2 84.3</td>
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*aNot determined*

Table 3  Effects of ascorbic acid on the retention (%) of 12.5, 25, 50, or 100 µM riboflavin under light.
<table>
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<tr>
<th>Quencher</th>
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<th>Slope (S)</th>
<th>S/I (mM)</th>
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<td>0.025</td>
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<td>27.17&lt;sup&gt;a&lt;/sup&gt;</td>
<td>2.338</td>
<td>0.086</td>
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Numbers with different superscripts in the same column are significantly different at $\alpha=0.05$.

Table 4  The intercepts and slopes from the regression lines in Figure 23 and Figure 24.
Figure 20  Degradation of riboflavin in H$_2$O and D$_2$O under light and in the dark.
Figure 21  Headspace oxygen depletion of various milk fat concentrations in cream containing 40 µM riboflavin under light for 3 h at 30°C.
Figure 22  Effects of 0, 0.44, 3.3, 6.7, and 10.0 mM sodium azide on riboflavin degradation.
Figure 23  Effects of 0, 10, 20, 40, 80, and 160 mM ascorbic acid on riboflavin in aqueous solution under light for 24 h at 30°C.
Figure 24 Effects of 0, 1, 5, 10, and 20 mM sodium azide on riboflavin in aqueous solution under light for 24 h at 30°C.
Figure 25  Relationship of the slope/intercept in Figure 24 to the concentrations of sodium azide.
CHAPTER 4

EFFECTS OF RIBOFLAVIN PHOTOSENSITIZED OXIDATION ON THE VOLATILE COMPOUNDS OF SOYMILK

4.1 Abstract

Soymilks with or without added riboflavin in serum bottles were stored under light or in dark at 20°C. The headspace oxygen and volatile compounds were determined by gas chromatography. Riboflavin had significant effects on the headspace oxygen depletion and volatile compounds formation in soymilk under light (P<0.05). Riboflavin did not have significant effects on the formation of volatile compounds and the depletion of headspace oxygen in the dark (P>0.05). The volatile compounds increased under light, but not in the dark as the added riboflavin increased. Storage temperature at 4°C or 20°C did not have significant differences in the effect of riboflavin on headspace oxygen depletion in soymilk under light. Hexanal, an important beany flavor compound, was identified as the major volatile compound in the riboflavin photosensitized soymilk. Singlet oxygen was involved in the formation of volatile compounds in soymilk under
light. Hexanal could be formed by singlet oxygen oxidation. Ascorbic acid, a quencher for singlet oxygen and the excited triplet sensitizer, significantly inhibited the formation of hexanal and total volatiles in soymilk under light.

4.2 Introduction

Soymilk is one of the traditional nutritious beverages used for thousands of years in oriental countries. In recent years, soymilk consumption among Western consumers has increased considerably due to the heightened awareness of its healthy benefits. Soy beverages were a $622 million market in 2003 and it is expected to grow to a $1.78 billion market by 2010, with the compounding annual growth rate of 17% in the USA (Savirly and Prakash 2004). However, due to the association of beany flavor with soymilk, the acceptance of soymilk in Western countries including USA is still hampered.

Hexanal is one of the major off-flavor compounds in soy products (Torres-Penaranda and Reitmeier 2001). Hexanal is a product from lipid oxidation, which can be intensified by the exposure of light, pro-oxidant metals (Marsili 1999), or enzymes such as lipoxygenase and hydroperoxylyase (Matoba and others 1985). Hexanal and pentanal were reported to be responsible for the cardboard-like flavors in milk (Hansen and Skibsted 2000; Kristensen and others 2001).

The oxidation of food components can be caused either by diradical triplet oxygen or non-radical singlet oxygen. Diradical triplet oxygen ($^3\text{O}_2$) readily reacts with radical
compounds in foods and is temperature dependent (Frankel 1985). Non-radical singlet oxygen \(^1\text{O}_2\) reacts directly with electron-rich compounds such as those containing double bonds. Singlet oxygen oxidation is not temperature dependent. Even at low temperatures, the oxidation rate of food components involving singlet oxygen is rather high (Min and others 1989). Linoleic acid oxidation caused by singlet oxygen is over 1450 times greater than that of triplet oxygen autoxidation (Rawls and VanSanten 1970; Frankel and others 1981).

The most important mechanism for the formation of singlet oxygen in foods is by photosensitized generation. Riboflavin, chlorophyll, myoglobin and some artificial food colorants are good photosensitizers (Foote and Denny 1968; Afonso and others 1999; Lledias and Hansberg 2000; King and Min 2002). In the presence of light and molecular oxygen, photosensitizers can generate singlet oxygen. One molecule of sensitizer may generate \(10^3\) to \(10^5\) molecules of singlet oxygen before becoming inactive (Min and others 1989; Kochevar and Redmond 2000).

Riboflavin is a water soluble photosensitizer (Min and Boff 2002). Upon absorbing energy from UV or visible light, riboflavin becomes an excited singlet state. An excited triplet state riboflavin is formed from the excited singlet state through the intersystem crossing mechanism. The excited triplet state riboflavin reacts with triplet (molecular) oxygen to form singlet oxygen (Type-II mechanism). The exited triplet riboflavin has a redox potential of 1.7 V at pH 7 (Lu and others 1999). It can abstract electron or hydrogen atoms from other substrates in foods such as polyunsaturated fatty acids and form free radicals of food compounds (Type-I mechanism).
Soymilk contains 2.86% protein, 1.53% fat, 0.27% ash, 1.53% carbohydrate and 93.81% of moisture (Rosenthal and others 2003). Riboflavin content in soymilk is about 3 ppm, which is comparable to the riboflavin content in cow’s milk (Kwok and others 1998). The effect of riboflavin on the formation of off-flavors in cow’s milk under light storage was thoroughly studied (Lee 2002). Pentanal, hexanal, heptanal and dimethyl disulfide were found to be increased significantly when milk was stored under light in the presence of increased levels of riboflavin.

Many attempts have been made to eliminate or minimize the beany flavor in soy products. Those include masking beany flavor with flavor materials (Lee and others 1990), process modification to produce soy product with lower or no beany flavors (Hunter and others 1983; Gupta 2003) and genetic modification of soybean varieties to reduce the enzymes responsible for the beany flavors (Ma and others 2001; Feng and others 2001). However, few studies were reported on the flavor changes of soymilk during storage under the supermarket retail conditions. The effect of riboflavin in soymilk on the formation of volatile compounds under light was not reported. The objectives of this research are (1) to study the effect of riboflavin on the volatile compounds in soymilk under light and (2) to determine the effects of ascorbic acid, a quencher for both the singlet oxygen and excited triplet riboflavin, on the formation of beany flavor compounds under light.
4.3 Materials and Methods

Materials

Soymilk containing 3 ppm riboflavin was purchased from a local organic food store in Columbus, Ohio. Riboflavin and ascorbic acid were purchased from Acros Organics (Jersey City, NJ). Water used in all sample preparations was purified by a Milli-Q purification system (Bedford, MA). Chemical standards of 1-pentenol, hexanal, 2-hexenal, heptanal, 2-heptenal, 1-octen-3-ol, and 2, 4-heptadienal were purchased from Sigma Chemical Co. (St. Louis, MO). The gas chromatography (GC) column was purchased from Restek Corporation (Bellefonte, PA). The 75 µm carboxen-polydimethylsiloxane (CAR/PDMS) solid phase microextraction fiber, fiber holder, glass GC inlet liner, 10 mL glass serum bottles, Teflon™-coated rubber septa, and aluminum crimp caps were purchased from Supelco, Inc. (Bellefonte, PA).

Sample preparation and storage

To study the effect of riboflavin concentrations on the depletion of headspace oxygen and the formation of volatiles in soymilk samples, soymilk with 0, 2, 4, 8, 16, 32, 64 and 128 ppm of added riboflavin was prepared. The original soymilk had 3 ppm riboflavin. A 1 mL aliquot of soymilk sample was pipetted into a 10 mL glass bottle and sealed air-tight with a Teflon™-coated rubber septum and an aluminum crimp cap. The
sample bottles were stored in duplicate in a light box at 1500 lux (Lee 2002) at temperatures of either 4°C or 20°C. The headspace oxygen and volatiles were analyzed after 24 and 48 h of storage.

The effects of light and temperature on the depletion of headspace oxygen and the formation of total volatiles in headspace of soymilk were studied by pipetting 1 mL soymilk supplemented with 50 ppm riboflavin into 10 mL glass sample bottles; the sealed bottles were stored under light or in the dark for up to 168 h. Samples were analyzed in duplicate for headspace oxygen after 24 and 48 h of storage. The total volatiles were analyzed after 0, 4, 8, 24, 48, and 168 h of storage.

To study the effect of ascorbic acid on the formation of volatiles in soymilk, ascorbic acid concentrations of 0, 10, 20, 40, 80, and 160 mM were added to soymilk containing 50 ppm riboflavin. Headspace volatiles were analyzed in duplicate by GC after the samples were stored under light for 24 or 48 h.

Analysis of headspace oxygen

Headspace oxygen in the sample bottles was analyzed by injecting a 100 µL headspace air sample into a HP 5890 GC (Avondale, PA) equipped with a stainless steel molecular sieve column (13X, 80:100; Alltech, Deerfield, IL) and a thermal conductivity detector (King and Min 2002). High purity helium (99.99%) was used as the carrier gas. The flow rate was at 40 mL/min. The GC oven temperature was maintained at 40°C. The injector port and detector temperatures were maintained at 120°C and 150°C,
respectively. Each sample was analyzed in duplicate. Oxygen content was quantified by an HP 3396A integrator (Yang and others 2002; King and Min 2002).

Analysis of headspace volatile compounds

Headspace volatiles were analyzed by solid phase microextraction (SPME) and gas chromatography (Steenson and others 2002; Lee and others 2003; Suratman and others 2004). Headspace volatile compounds were isolated by SPME with a 75 μm Carboxen/polydimethylsiloxane (CAR/PDMS) fiber. The fiber was exposed to the bottle headspace for 30 minutes while the bottle was incubated in a 50°C water bath (Lee 2002).

The extracted volatiles were desorbed in the GC injection port for 3 minutes at 250°C. The injection port was fitted with a 0.75 mm internal diameter splitless glass liner. Volatile compounds were separated using an Rtx-5 (Crossbond 5% diphenyl-95% dimethyl polysiloxane) column (30 m x 0.32 mm x 0.1 μm film thickness) and detected with a flame ionization detector at Hewlett-Packard 6890 GC (Wilmington, DE) using hydrogen as the carrier gas. The GC oven temperature was programmed to hold at 60°C for 3 minutes, increase to 120°C at 4°C/minute, increase to 240°C at 10°C/minute, and then hold at 240°C for 2 minutes (Lee 2002).

The HP 5970 series mass spectrometer was used for the identification of volatile compounds of soymilk. The condition for GC/MS was the same as the GC analysis as described above. The mass spectra were obtained at 70 eV and 220°C ion source temperature. The compounds were identified by the combination of mass spectra and the gas chromatographic retention times of standard compounds.
Analysis of data

The data were statistically analyzed using the T-test and the Microsoft Office Excel Program.

4.4 Results and Discussion

Reproducibility of headspace oxygen and headspace volatile analysis

The coefficient of variations for headspace oxygen analysis and total headspace volatile analysis for 6 replicate soymilk samples was 2.24% and 3.31%, respectively (data not shown). The coefficients of variations were considered acceptable for the study of soymilk oxidation by measuring the changes of headspace oxygen and volatile compounds in sample bottles.

Effect of riboflavin on the headspace oxygen of soymilk under light

The effects of the 0, 2, 4, 8, 16, 32, 64 and 128 ppm added riboflavin on the depletion of headspace oxygen (%) in soymilk after 48 h under light are shown in Table 5. Riboflavin had a significant effect on the headspace oxygen depletion in soymilk under light (P<0.05). The more riboflavin added to the soymilk directly correlated with a lower concentration of headspace oxygen in the sealed sample bottles. The headspace oxygen content of soymilk with added 50 ppm riboflavin decreased from 21% (the oxygen content of air) to 13.18% during storage of 48 h under light. The oxygen that dissipated from the gas tight sample bottle was probably due to the reaction between
oxygen and soymilk. Riboflavin in soymilk accelerated the reaction between oxygen and soymilk components under light, resulting in the depletion of the headspace oxygen. The headspace oxygen contents of soymilk with 0, 2, 4, 8, 16, 32, 64, or 128 ppm added riboflavin in dark were essentially the same at about 20.7%. Riboflavin concentration did not have a significant effect on the headspace oxygen depletion when soymilk was stored in the dark for 48 h (Table 5).

Identification of volatile compounds in soymilk with or without the added 50 ppm riboflavin

The effects of the added 50 ppm riboflavin on the headspace volatile compounds in soymilk under light for 24 h at 20°C are shown in Figure 26. The figure shows that the added 50 ppm riboflavin increased the quantity and the numbers of volatile compounds during storage for 24 h under light. It was very difficult to identify the compounds in the soymilk under light for 24 h by mass spectrometry due to their low concentrations. Therefore, the samples were stored for 168 h under light to increase the concentration of volatile compounds for identification by mass spectrometry. The compounds were identified by the combination of mass spectrometry and gas chromatographic retention time of authentic compounds. The gas chromatograms of soymilk stored for 168 h under light are not shown. The identified volatile compounds and their changes during storage of soymilk under light for 168 h are shown in Table 6. Pentane and pentanol were the major compounds in fresh soymilk and did not change significantly in soymilk stored under light for 168 h. Hexanal was the predominant volatile compound in soymilk under
light (Table 6). The compounds 2-heptanone, 2-heptenal, 1, 3-dimethylbenzene, isobutyl butyrate, 1-octen-3-ol, 3-octen-2-one, ethyl 5-hydroxydecanonate, and nonanal were also identified in the soymilk stored for 168 h under light.

Effects of riboflavin on pentane, hexanal, and the total volatile compounds

The effects of the 0, 2, 4, 8, 16, 32, 64, or 128 ppm added riboflavin on the levels of pentane, hexanal, and total volatile compounds in soymilk are summarized in Table 7. As the added riboflavin increased from 0, 2, 4, 8, 16, 32, 64, to 128 ppm, hexanal and total volatile compounds increased significantly (P<0.05). Hexanal was the most abundant volatile compound in soymilk headspace after storage under light for 24 or 48 h, as shown in Table 7. Pentane was one of the major volatile compounds in fresh soymilk. Light and the added riboflavin had no significant effect on the concentration of pentane in soymilk headspace after 24 and 48 h of storage.

Effect of the added 50 ppm riboflavin on the headspace oxygen in soymilk

The effect of added 50 ppm riboflavin on the headspace oxygen (%) of soymilk under light and in dark at 4°C or 20°C is shown in Table 8. The headspace oxygen of soymilk with added 50 ppm riboflavin was 17.99% after 24 h and 14.05% after 48 h under light at 20°C. The headspace oxygen of soymilk with added 50 ppm riboflavin in dark at 20°C was 20.70% and 20.56% after 24 and 48 h, respectively. The added 50 ppm riboflavin had a significant effect on the headspace oxygen in soymilk stored under light for 24 and 48 h at 4°C or 20°C (P<0.05). The added 50 ppm riboflavin did not have
significant effects on the headspace oxygen in soymilk during the storage for 24 and 48 h at 4°C or 20°C in the dark (P>0.05). The storage time of 24 and 48 h had significant effects on the headspace oxygen of soymilk without the added 50 ppm riboflavin only under light, not in the dark. The soymilk used in this research contained 3 ppm riboflavin. The 3 ppm riboflavin in soymilk had a significant effect on the headspace oxygen of soymilk during storage for 24 or 48 h under light. The effects of storage temperatures of 4°C and 20°C on the headspace oxygen of soymilk with added 50 ppm riboflavin for 24 h or 48 h were not significant (Table 8). Storage temperature did not have significant effects on the oxidation of foods when singlet oxygen was involved (Min and Boff 2002). The effect of the added 50 ppm riboflavin on the headspace oxygen in soymilk indicated that singlet oxygen oxidation was involved in the soymilk oxidation under light. Riboflavin produces singlet oxygen under light in the presence of atmospheric triplet oxygen. The added 50 ppm riboflavin, which produces singlet oxygen under light (King and Min 2002; Min and Boff 2002), had a significant effect on the headspace oxygen depletion of soymilk under light (P<0.05).

Effects of the added 50 ppm riboflavin on the pentane, hexanal and total volatile compounds of soymilk under light and in dark

The addition of 50 ppm riboflavin to soymilk had significant effects on the formation of headspace volatiles under light at α=0.05 during 24 h under light. The total volatile increased 12.5 and 30 times after soymilk with added 50 ppm riboflavin stored under light for 24 and 48 h, respectively (Table 9). Light did not have a significant effect on the content of pentane in soymilk headspace during the 0, 4, 8, 24, 48, or 168 h of
storage. Light played an important role on the formation of hexanal and total volatiles in soymilk containing riboflavin. The change of hexanal in the headspace of soymilk with or without the added 50 ppm riboflavin in dark was not significant during storage for 168 h.

The hexanal content in soymilk headspace without the added 50 ppm riboflavin increased as the storage time increased from 0, 4, 8, 24, 48, to 168 under light. It should be noted that the soymilk without the added 50 ppm riboflavin also contained 3 ppm riboflavin. The naturally present 3 ppm riboflavin in soymilk might be responsible for the increase of hexanal in soymilk headspace during storage under light. However, the addition of 50 ppm riboflavin increased the hexanal content from 1733 to 7925 in electronic counts during storage for 48 h (Table 9). The added 50 ppm riboflavin increased hexanal and total volatiles in the soymilk headspace under light, not in the dark (Table 9).

Mechanisms of hexanal and 2-heptenal formation from linoleic acid by singlet oxygen

Hexanal has a strong grassy and beany flavor (Wang and others 1998; Torres-Penaranda and Reitmeier 2001; Mizutani and Hashimoto 2004). Research has reported that all panelists could detect the presence of the beany flavor in soymilk when the hexanal concentration was 25 ppm (Hashim and Chaveron 1995). Hexanal in soymilk can be formed by the combination of Type-I and Type-II mechanisms in riboflavin photosensitized system. Though hexanal was reported to be mainly formed from linoleic acid through the triplet oxygen autooxidation in soybean oil by Type-I mechanism (Min
and Boff 2002), hexanal in soymilk could be formed through the singlet oxygen oxidation of linoleic acid by Type-II mechanism in photosensitized oxidation as shown in Figure 27. The compound 2-heptenal, a unique product from the singlet oxygen oxidation of linoleic acid as shown in Figure 28, was present in soymilk under light but not in soymilk stored in the dark. This result suggests that singlet oxygen oxidation was involved for volatile compound formation in soymilk under light. Singlet oxygen could not produce pentane from fatty acids in soymilk. Therefore, the pentane content did not increase in the soymilk headspace during storage under light.

Effect of ascorbic acid on the formation of volatile compounds in soymilk under light

The effects of 0, 10, 20, 40, 80, and 160 mM ascorbic acid on the concentration of pentane, hexanal, and total volatiles in soymilk headspace with added 50 ppm riboflavin stored under light for 24 and 48 h are shown in Table 10. Ascorbic acid significantly reduced the level of hexanal and total volatile compounds, but had no effect on pentane levels under light. Ascorbic acid is an excellent singlet oxygen quencher (Min and Boff 2002) and minimized the hexanal formation by singlet oxygen quenching in soymilk. Although hexanal can be formed by singlet oxygen oxidation in soymilk, pentane cannot.
4.5 Conclusions

Riboflavin had significant effects on the depletion of headspace oxygen and the formation of total volatile compounds in soymilk samples stored under light. Hexanal, which is associated with beany flavor, was the most abundant volatile compound formed in soymilk headspace under light. Hexanal is formed by singlet oxygen oxidation of linoleic acid in soymilk. The singlet oxygen is formed by the riboflavin photosensitized reaction. Fresh soymilk with no beany flavor could produce beany flavor by the singlet oxygen oxidation in soymilk during storage under light, not in the dark. Ascorbic acid could inhibit the formation of hexanal and total volatile in soymilk under light. Soymilk with or without added riboflavin did not increase the total volatile compounds after 48 h in the dark. Soymilk should be stored in the dark and can be fortified with ascorbic acid to minimize the formation of undesirable volatile compounds.

4.6 References


Table 5  Effect of added riboflavin on the depletion of headspace oxygen (%) in soymilk under light for 48 h.

<table>
<thead>
<tr>
<th>Added riboflavin in soymilk (ppm)</th>
<th>0</th>
<th>2</th>
<th>4</th>
<th>8</th>
<th>16</th>
<th>32</th>
<th>64</th>
<th>128</th>
</tr>
</thead>
<tbody>
<tr>
<td>Headspace oxygen (%) under light</td>
<td>19.47&lt;sup&gt;a&lt;/sup&gt;</td>
<td>18.78&lt;sup&gt;a&lt;/sup&gt;</td>
<td>16.74&lt;sup&gt;b&lt;/sup&gt;</td>
<td>15.33&lt;sup&gt;c&lt;/sup&gt;</td>
<td>14.40&lt;sup&gt;d&lt;/sup&gt;</td>
<td>14.20&lt;sup&gt;d&lt;/sup&gt;</td>
<td>13.98&lt;sup&gt;d&lt;/sup&gt;</td>
<td>13.18&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>Headspace oxygen (%) in dark</td>
<td>20.81&lt;sup&gt;a&lt;/sup&gt;</td>
<td>20.75&lt;sup&gt;a&lt;/sup&gt;</td>
<td>20.84&lt;sup&gt;a&lt;/sup&gt;</td>
<td>20.76&lt;sup&gt;a&lt;/sup&gt;</td>
<td>20.72&lt;sup&gt;a&lt;/sup&gt;</td>
<td>20.70&lt;sup&gt;a&lt;/sup&gt;</td>
<td>20.66&lt;sup&gt;a&lt;/sup&gt;</td>
<td>20.65&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Different superscripts in the same row are significantly different at α=0.05.
<table>
<thead>
<tr>
<th>Compounds</th>
<th>0 h</th>
<th>Dark</th>
<th>Light</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pentane</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Pentanal</td>
<td>-</td>
<td>-</td>
<td>+++</td>
</tr>
<tr>
<td>1-Pentanol</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Hexanal</td>
<td>-</td>
<td>+</td>
<td>+++++++</td>
</tr>
<tr>
<td>Dimethylbenzene</td>
<td>-</td>
<td>-</td>
<td>+++</td>
</tr>
<tr>
<td>2-Heptanone</td>
<td>-</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td>Isobutyl butyrate</td>
<td>-</td>
<td>-</td>
<td>+++</td>
</tr>
<tr>
<td>2-Heptenal</td>
<td>-</td>
<td>-</td>
<td>+++</td>
</tr>
<tr>
<td>1-Octen-3-ol</td>
<td>-</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td>Unknown</td>
<td>-</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td>3-Octen-2-one</td>
<td>-</td>
<td>-</td>
<td>++</td>
</tr>
<tr>
<td>Ethyl 5-hydroxydecanoate*</td>
<td>-</td>
<td>-</td>
<td>+++</td>
</tr>
<tr>
<td>Nonanal</td>
<td>-</td>
<td>-</td>
<td>++</td>
</tr>
</tbody>
</table>

*a*: The compounds were listed according to the gas chromatographic retention times.

*: Tentative identification

-: The gas chromatographic peaks are so small such that the HP electronic integrator can not integrate or the peaks were not present at all.

+: the peak area <100; ++: the peak area is between 101~500; +++: the peak area is between 501~1000; ++++++++: the peak area is > 5000.

Table 6  Volatile compounds in soymilk headspace with added 50 ppm riboflavin under light or dark at 4°C for 168 h.
Volatile compounds are expressed in electronic counts of gas chromatographic peaks.
Superscripts with different letters in the same row are significantly different at α=0.05

Table 7  Effects of added riboflavin on the pentane, hexanal, and total volatile of soymilk under light for 24 and 48 h at 20°C.
### Table 8  Effect of added 50 ppm riboflavin on the headspace oxygen (%) of soymilk under light or in dark at 4°C or 20°C.

<table>
<thead>
<tr>
<th>Riboflavin (ppm)</th>
<th>Storage</th>
<th>Storage at 4°C (h)</th>
<th>Storage at 20°C (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
<td>24</td>
</tr>
<tr>
<td>0</td>
<td>Light</td>
<td>20.91&lt;sup&gt;a&lt;/sup&gt;</td>
<td>20.25&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>0</td>
<td>Dark</td>
<td>20.88&lt;sup&gt;a&lt;/sup&gt;</td>
<td>20.75&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>50</td>
<td>Light</td>
<td>20.85&lt;sup&gt;a&lt;/sup&gt;</td>
<td>18.25&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>50</td>
<td>Dark</td>
<td>20.86&lt;sup&gt;a&lt;/sup&gt;</td>
<td>20.81&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Superscripts with different letters in the same column are significantly different at α =0.05
### Table 9: Effect of added riboflavin on pentane, hexanal, and total volatile compounds in electronic counts of soymilk under light or in dark.

<table>
<thead>
<tr>
<th>Riboflavin (ppm)</th>
<th>Storage</th>
<th>Compound</th>
<th>Storage under light or in dark at 20°C (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>0</td>
<td>Light</td>
<td>Pentane</td>
<td>133&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Hexanal</td>
<td>12&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Total</td>
<td>471&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>0</td>
<td>Dark</td>
<td>Pentane</td>
<td>123&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Hexanal</td>
<td>11&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Total</td>
<td>488&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>50</td>
<td>Light</td>
<td>Pentane</td>
<td>184&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Hexanal</td>
<td>18&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Total</td>
<td>410&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>50</td>
<td>Dark</td>
<td>Pentane</td>
<td>170&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Hexanal</td>
<td>24&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Total</td>
<td>350&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Volatile compounds are expressed in electronic counts of gas chromatographic peaks.

Superscripts with different letters in the same row are significantly different at $\alpha=0.05$. 

Table 9: Effect of added riboflavin on pentane, hexanal, and total volatile compounds in electronic counts of soymilk under light or in dark.
<table>
<thead>
<tr>
<th>Ascorbic acid (mM)</th>
<th>0</th>
<th>10</th>
<th>20</th>
<th>40</th>
<th>80</th>
<th>160</th>
</tr>
</thead>
<tbody>
<tr>
<td>24 h</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pentane</td>
<td>269a</td>
<td>312a</td>
<td>192a</td>
<td>186a</td>
<td>152a</td>
<td>166a</td>
</tr>
<tr>
<td>Hexanal</td>
<td>2580a</td>
<td>1809b</td>
<td>1057c</td>
<td>221d</td>
<td>107d</td>
<td>126d</td>
</tr>
<tr>
<td>Total</td>
<td>5077a</td>
<td>3004b</td>
<td>2047c</td>
<td>1587d</td>
<td>1134e</td>
<td>852e</td>
</tr>
<tr>
<td>48 h</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pentane</td>
<td>154a</td>
<td>174a</td>
<td>134a</td>
<td>163a</td>
<td>113a</td>
<td>222a</td>
</tr>
<tr>
<td>Hexanal</td>
<td>7576a</td>
<td>4673b</td>
<td>2160c</td>
<td>655d</td>
<td>142e</td>
<td>95e</td>
</tr>
<tr>
<td>Total</td>
<td>10799a</td>
<td>6396b</td>
<td>3335c</td>
<td>1607d</td>
<td>1601d</td>
<td>1098d</td>
</tr>
</tbody>
</table>

Volatile compounds are expressed in electronic counts of gas chromatographic peaks.
Superscripts with different letters in the same row are significantly different at \( \alpha = 0.05 \)

Table 10  Effects of ascorbic acid on the pentane, hexanal, and total volatile compounds of soymilk with added 50 ppm riboflavin under light for 24 or 48 h.
Figure 26  The effects of added 50 ppm riboflavin on the gas chromatograms of headspace volatile compounds of soymilk under light for 24 h at 20°C

(Top: without riboflavin addition, Bottom: with riboflavin addition).
Figure 27  Mechanism for hexanal formation from linoleic acid by singlet oxygen.
Figure 28  Mechanism for 2-heptenal formation from linoleic acid by singlet oxygen.
CHAPTER 5

PHOTOSENSITIZING EFFECT OF RIBOFLAVIN, LUMIFLAVIN, AND LUMICRO…

LUMICRO… ON SOYMILK FLAVOR STABILITY

5.1 Abstract

Lumichrome and lumiflavin were formed from riboflavin under light, under influence of pH. Lumichrome was the only major product of riboflavin at neutral or acidic pH. Lumiflavin was also formed from riboflavin in basic pH. The maximum concentration of lumiflavin from 100 µM riboflavin at pH 8.5 was 30.9 µM after 2 h of exposure at 1500 lux. The maximum concentration of lumichrome formed from 100 µM riboflavin at pH 4.5, 6.5, or 8.5 was 79.9, 58.7, and 73.1 µM, respectively, after 8, 6, or 2 h of light exposure. The formation of lumichrome and lumiflavin from riboflavin was due to the Type-I mechanism of riboflavin photosensitized reaction. Singlet oxygen was also involved in the photosensitized degradation of lumiflavin and lumichrome. The reaction rates of riboflavin, lumiflavin, and lumichrome with singlet oxygen were $9.66 \times 10^8 \text{ M}^{-1}\text{s}^{-1}$, $8.58 \times 10^8 \text{ M}^{-1}\text{s}^{-1}$ and $8.21 \times 10^8 \text{ M}^{-1}\text{s}^{-1}$, respectively. The headspace oxygen depletion
and headspace volatile formation were significant in soymilk containing lumichrome or lumiflavin under light (P<0.05); they were insignificant (P>0.05) in dark. Ascorbic acid could inhibit the total volatile changes of soymilk under light. Soymilk should be protected from light to prevent the photodegradation of riboflavin and the oxidation of soymilk.

5.2 Introduction

Riboflavin exists in milk, eggs, meats, vegetables, and many other food products (Szczesniak and others 1971; Gliszczynska and Koziolowa 1999; USDA 2003). Because of its complex photochemical properties (Kim and others 1993; Crank and Pardijanto 1995; Li and Min 1998; Edwards and others 1999; Viteri and others 2003; Criado and others 2003), riboflavin has been extensively studied as a photosensitizer in food (Huang and others 2004a) and biological systems (Lucius and others 1998; Grzelak and others 2001; Hlavinka and others 2003). In addition to being an effective photosensitizer to produce singlet oxygen, riboflavin is also a good reactant for singlet oxygen (Min and Boff 2002). When exposed to light, riboflavin quickly photodegraded in aqueous solution (Huang and others 2004b). Similarly, the degradation of riboflavin in foods stored under light was significant. Over seventy-five % of the riboflavin in cheese was degraded within 100 days. However, riboflavin degradation in food items was insignificant when stored in dark (Kristensen and others 2001). Depending on the pH of the riboflavin solution, riboflavin was reported to be photodegraded into lumichrome, lumiflavin, and others (Ansari and others 2004; Ahmad and others 2004b).
While it is well known that riboflavin is an effective photosensitizer in food systems (Yang and others 2002; Min and Boff 2002; Hlavinka and others 2003), no research has determined whether the riboflavin degradation products, lumichrome and lumiflavin, can be photosensitizers in food. Therefore, the objectives of this research are 1) to study the effect of pH on the photodegradation of riboflavin by separation and identification the degradation products; 2) to determine the reaction rate between singlet oxygen and riboflavin, lumiflavin, and lumichrome; 3) to study the effects of riboflavin, lumiflavin, and lumichrome as the photosensitizers on soymilk flavor stability; and 4) to study the effect of ascorbic acid on inhibiting the flavor changes of soymilk under light.

5.3 Materials and Methods

Materials

Soymilk containing 3 ppm riboflavin was purchased from a local organic food store in Columbus, Ohio. Riboflavin was purchased from Acros Organics (Jersey City, NJ). Sodium azide, lumichrome, and lumiflavin were purchased from Sigma Chemical Co. (St. Louis, MO). Water used in sample preparations was purified by a Milli-Q purification system (Millipore Co., Bedford, MA). Serum bottles (10 mL), aluminum caps, and Teflon™-coated septa were purchased from Supelco, Inc. (Bellefonte, PA).
Sample preparations

A 2 mL aliquot of the 100 µM riboflavin solution in H₂O was pipetted into a 10 mL serum bottle and sealed to be air-tight with a Teflon™-coated rubber septum and an aluminum crimp cap. The sample bottles were then stored under light for up to 72 h. The light intensity of the storage box was at 1500 lux (Lee 2002; Huang and others 2004a). Duplicate samples were taken out of the light box at 2, 4, 8, 16, 24, 48, and 72 h for analysis of riboflavin concentration and its degradation products.

To study the effect of pH on the riboflavin degradation and the formation of degraded products, riboflavin solutions at concentration of 100 µM were made in 100 mM phosphate buffer at pH 4.5, 6.5, and 8.5. Samples (2 mL) were then sealed to be air-tight in 10 mL serum bottles for storage under light up to 72 h. The changes of riboflavin and its degradation products were determined by an HPLC method.

To detect the involvement of singlet oxygen in photodegradation, riboflavin, lumiflavin, and lumichrome solutions were prepared at 50 µM. Sodium azide, a well known singlet oxygen quencher, was added at a concentration of 5 mM. Samples (2 mL) with or without sodium azide were sealed to be air-tight in 10 mL serum bottles and stored under light for up to 24 h. The change of riboflavin, lumiflavin, and lumichrome was determined by the HPLC method.

To determine the reaction rate constant between riboflavin, lumichrome or lumiflavin, and singlet oxygen, aqueous solutions of these compounds were made at 12.5, 25, 50, and 100 µM, respectively. After being stored under light for 24 h, the residual concentrations of riboflavin, lumiflavin, and lumichrome were determined by HPLC method. The steady-state kinetic equation for singlet oxygen and the excited flavin
quenching (Foote 1979; Yang and others 2002) was then used to calculate the respective reaction rate constants of singlet oxygen with riboflavin, lumiflavin, and lumichrome.

To study the effect of riboflavin, lumiflavin, and lumichrome on the flavor stability of soymilk, soymilk samples were supplemented with 100 µM of each. A 2 mL aliquot of soymilk was pipetted into a 10 mL glass bottle and sealed air-tight with a Teflon™-coated rubber septum and an aluminum crimp cap. The sample bottles were stored in a light box at 1500 lux (Lee 2002; Huang and others 2004a) at 4°C. The headspace oxygen and total volatiles were analyzed in duplicate after 24, 48, and 72 h of storage.

To study the effect of ascorbic acid on the changes of volatile compounds, soymilk was first prepared to contain 100 µM of riboflavin, lumichrome, or lumiflavin. Ascorbic acid was then added at 50 mM. The total volatile compounds in the headspace of soymilk were analyzed with SPME/GC/FID after the samples were stored under light at 4 °C for up to 72 h.

Analysis of riboflavin and its photodegradation products

An HPLC (HP 1100; Hewlett-Packard, Wilmington, DE) equipped with an Aqua C-18 column (5 µ, 150 mm x 4.60 mm) (Phenomenex, Torrance, CA) was used to monitor the change of riboflavin and its degradation products. Samples removed from the light box were filtered with a 0.2 µm membrane filter before injection. The injection volume was 10 µL. The flow rate of the mobile phase (methanol:water=3:7 (v/v)) was 1.0 mL/min. An HP 1100 Diode Array Detector was used to measure the absorbance of
riboflavin and its degraded products. The concentrations of riboflavin, lumichrome, and lumiflavin were calculated using a standard linear line of HPLC peaks of different concentrations of respective standard chemicals in aqueous solution.

Riboflavin degradation products were identified by the combination of the retention time and UV-Vis spectra comparison against the authentic standard chemicals, and the HPLC/MS analysis.

Analysis of headspace oxygen

Headspace oxygen in the sample bottles was analyzed by injecting a 100 µL headspace air sample into a HP 5890 GC (Avondale, PA), equipped with a stainless steel molecular sieve column (13X, 80:100; Alltech, Deerfield, IL) and a thermal conductivity detector (King and Min 2002). High purity helium (99.99%) was used as the carrier gas. The flow rate was at 40 mL/min. The GC oven temperature was maintained at 40°C. The injector port and detector temperatures were maintained at 120°C and 150°C, respectively. Each sample was analyzed in duplicate. Oxygen contents were quantified by an HP 3396A integrator (Yang and others 2002; King and Min 2002).

Analysis of headspace volatile compounds

Headspace volatiles were analyzed by solid phase microextraction (SPME) and gas chromatography (Lee 2002; Steenson and others 2002; Lee and others 2003; Suratman and others 2004). Headspace volatile compounds were isolated by SPME with a 75 µm Carboxen/polydimethylsiloxane (CAR/PDMS) fiber. The fiber was exposed to
the bottle headspace for 30 minutes while the bottle was incubated in a 40°C water bath (Steenson and others 2002; Lee and others 2003).

The extracted volatiles were desorbed in the GC injection port for 3 minutes at 250°C. The injection port was fitted with a 0.75 mm internal diameter splitless glass liner. Volatile compounds were separated using an Rtx-5 (Crossbond 5% diphenyl-95% dimethyl polysiloxane) column (30 m x 0.32 mm x 0.1 µm film thickness) and detected with a flame ionization detector at Hewlett-Packard 6890 GC (Wilmington, DE) using hydrogen as the carrier gas. The GC oven temperature was programmed to hold at 60°C for 3 minutes, increase to 120°C at 4 °C/minute, increase to 240°C at 10°C/minute, and then hold at 240°C for 2 minutes (Steenson and others 2002; Lee and others 2003).

Analysis of data

All treatments were duplicated in this study. Data were analyzed using the Microsoft Office Excel Program. Comparisons for mean value differences were done by T test. A P-value of ≤0.05 was considered significant.

5.4 Results and Discussion

Identification of the riboflavin photodegradation products in aqueous solution

Aqueous solution of riboflavin (100 µM) was prepared and stored under light for up to 72 h. Sample bottles wrapped with aluminum foil were designated as the samples
stored in the dark. The samples were analyzed by an HPLC method. Riboflavin was quickly degraded under light but remained unchanged in dark throughout the study (Huang and others 2004b). As the riboflavin peak decreased under light, a peak with a retention time of 35.6 minutes increased progressively. The 35.6 minute retention time of the peak was the same as that of the lumichrome standard (Figure 29). The UV-Vis spectrum of the peak was the same as that of the lumichrome standard (Figure 30). HPLC/MS analysis indicated that the degradation product had \textit{m/z} 243 \([\text{M+H}]^+\) (Figure 31), which represented the molecular cation of lumichrome. The combined results of retention time, UV-Vis spectrum comparison (Zeng and others 2003), and the HPLC/MS analysis indicated that the riboflavin degradation product with the retention time of 35.6 minutes was lumichrome.

Ahmad \textit{et al} reported that lumichrome was the major riboflavin degradation product in neutral to acidic pHs. When the pH of the solution was between 7 and 9, lumiflavin was also one of the major degradation products. Formylmethylflavin (FMF) and carboxymethylflavin (CMF) were reported as the other minor products (Ahmad and others 2004a).

Effect of pH on the riboflavin photodegradation

To study the effects of pH on the photodegradation products of riboflavin, phosphate buffers (100 mM) at pH 4.5, 6.5 and 8.5 were used to prepare riboflavin solutions at 100 \(\mu\text{M}\). After being stored under light for various length of time, the samples were analyzed by HPLC. Riboflavin was quickly degraded under light. Lumichrome was the only
major photodegradation product of riboflavin under neutral to acidic pHs, which is consistent with previous research (Woodcock and others 1982; Ahmad and others 2004a). At pH 4.5, the maximum lumichrome concentration (79.9 µM) was reached after 8 h under 1500 lux light. At pH 6.5, the maximum lumichrome concentration (58.7 µM) was reached after 6 h of storage under light. At pH 8.5, the maximum lumichrome concentration (73.1 µM) was reached after only 2 h under light. In addition to lumichrome, lumiflavin was also detected as the riboflavin photodegradation product at pH of 8.5 (Table 11). The maximum concentration 30.9 µM was reached after 2 h under light. Both lumichrome and lumiflavin started to degrade after reaching their respective maximum concentrations at various pHs (Table 11). This suggested the unstable nature of lumiflavin and lumichrome under light. Lumichrome seemed to be relatively more stable in lower pH solutions than that in a higher pH solution.

Effect of sodium azide on the photostability of riboflavin, lumichrome, and lumiflavin

To determine the involvement of singlet oxygen in the photodegradation of riboflavin, lumichrome, and lumiflavin, sodium azide, a well known singlet oxygen quencher, was added to the solution. If the addition of sodium azide could increase the stability of riboflavin, lumichrome, and lumiflavin in solution, singlet oxygen was involved in the photodegradation. Sodium azide in 50 µM riboflavin, lumiflavin, or lumichrome solution was added at 5 mM. Sodium azide greatly increased the stability of riboflavin, lumichrome, and lumiflavin under light (Table 12). After 8 h under light, riboflavin was nearly undetectable without sodium azide, while 56.2% of the riboflavin still remained with 5 mM sodium azide in the solution. Similarly, a higher percentage of
lumiflavin and lumichrome remained in the solution when sodium azide was included in the samples (Table 12). These results suggested that singlet oxygen was involved in the photodegradation of riboflavin, lumichrome, and lumiflavin. The lifetime of singlet oxygen in D$_2$O is about 13 times longer than that in H$_2$O. The longer lifetime of singlet oxygen in D$_2$O increases the reaction between singlet oxygen and riboflavin, resulting in higher riboflavin degradation. Faster degradation of riboflavin prepared in D$_2$O as opposed to H$_2$O also suggested the involvement of singlet oxygen in the photodegradation of riboflavin (Huang and others 2004b).

Mechanism of lumiflavin and lumichrome formation from riboflavin

The formation of lumichrome and lumiflavin from riboflavin requires synchronous fragmentation of a N (10)-C(1′) and a C(2′)-H bond in cis-periplanar conformation. Dealkylation involves per-attack at the flavin nucleus, thus favoring reactions at the C (2′) of the N (10) side chain and six member cyclic transition states (Gladys and Knappe 1974). These occur predominantly through the excited triplet state of riboflavin (Allen and Parks 1979). As suggested in Figure 32, the formation of lumiflavin and lumichrome was through the Type-I mechanism. The Type-II mechanism may be involved after lumichrome and lumiflavin are formed from riboflavin, though the direct involvement of excited triplet riboflavin ($^3$RF*) in Type-II mechanism could not be ruled out.
Reaction rate constant between singlet oxygen and riboflavin, lumichrome, and lumiflavin

Lumichrome and lumiflavin were identified as the major photodegradation products from riboflavin under light. Based on their molecular structure, it is expected that those molecules are also good reactants for singlet oxygen. As described previously, singlet oxygen was involved in the photodegradation of riboflavin, lumiflavin, and lumichrome. The steady-state kinetic equation for singlet oxygen and the excited flavin quenching (Foote 1979; Yang and others 2002) can be used to study the reaction rate constant between singlet oxygen and the flavin involved in the system:

\[
\left\{ \frac{d[FO_2]}{dt} \right\}^{-1} = K^{-1} \left( 1 + \frac{k_Q[O]}{k_o[3O_2]} \right) \left( 1 + \frac{(k_{ox-Q})[O] + k_d}{k_r} \times [F]^{-1} \right)
\]

where \([FO_2]\) is the concentration of oxidized flavins; \(K\) is the quantum yield of intersystem crossing; \(k_Q\) is the reaction constant for triplet flavin quenching; \([O]\) is the quencher concentration; \(k_o\) is the reaction constant for singlet oxygen formation by energy transfer from excited triplet flavin to triplet oxygen; \([3O_2]\) is the triplet oxygen concentration; \(k_q\) is the reaction constant of singlet oxygen physical quenching; \(k_{ox-Q}\) is the reaction constant of singlet oxygen chemical quenching; \(k_d\) is the reaction rate for singlet oxygen decay in a solvent; \(k_r\) is the reaction constant between flavins and singlet oxygen; and \([F]\) is the initial concentration of riboflavin, lumiflavin, and lumichrome.

When there is no quencher added in an experimental system, the equation can be simplified to the following (Foote 1979; Jung and others 1995; Lee and others 1997):

\[
\left\{ \frac{d[FO_2]}{dt} \right\}^{-1} = K^{-1} \left( 1 + \frac{k_d}{k_r} \times [F]^{-1} \right)
\]
From a plot of \([\text{FO}_2]^{-1}\) against \([\text{F}]^{-1}\), the ratio of the slope to intercept is equal to \(k_d/k_r\). Because the singlet oxygen decay rate \(k_d\) in aqueous solution is known, \(k_r\), the reaction rate constant between flavin and singlet oxygen, can be calculated (Foote 1979).

To determine the reaction rate constant between riboflavin, lumichrome or lumiflavin, and singlet oxygen, aqueous solutions of these compounds were made at concentrations of 12.5, 25, 50, and 100 µM, respectively. After being stored under light for 24 h, the residual concentrations of riboflavin, lumichrome, and lumiflavin were determined with an HPLC method. The reciprocal plots of the degraded flavin concentration against the initial flavin concentrations are shown in Figure 33. The slope and intercept for the regression lines in Figure 33 are summarized in Table 13. The singlet oxygen decay rate \(k_d\) in water was reported at 2.56 x 10\(^5\) s\(^{-1}\) (Rodgers and Snowden 1982; Hurst and Schuster 1983). Thus the reaction rate (\(k_r\)) between flavin and singlet oxygen can be calculated as 2.56 x 10\(^5\) s\(^{-1}\)/S/I. These results are shown in the last column of Table 13.

The results prove that riboflavin had a slightly higher reaction rate constant than lumichrome and lumiflavin. The \(k_r\) for riboflavin is consistent with previous research (Huang and others 2004; Haag and Mill 1987). This is potentially the first report of the reaction constant between singlet oxygen and lumichrome and lumiflavin.
Effect of riboflavin, lumiflavin, and lumichrome on the headspace oxygen of soymilk under light

Considering the quick degradation of riboflavin in aqueous solution under light, the degradation products from riboflavin may also act as the photosensitizers, which in turn cause the oxidative deterioration of food products. To test this hypothesis, soymilk was used as the model system and compared the effect of riboflavin, lumiflavin and lumichrome on the headspace oxygen of soymilk under light and in dark.

Riboflavin, lumiflavin, or lumichrome was added to soymilk at the final concentration of 100 µM. Soymilk without the addition of the flavins was used as the control. Results in Figure 34 showed the changes of headspace oxygen among soymilk supplemented with 100 µM riboflavin, lumiflavin, or lumichrome and stored under light for up to 72 h. Light played a significant role on the headspace oxygen depletion in soymilk. Supplementation of riboflavin, lumichrome, or lumiflavin did not influence the change of headspace oxygen in soymilk bottles when they were stored in the dark. After being stored in dark for 48 h, the headspace oxygen concentration in the samples stayed between 20.2% and 20.7%. When soymilk was stored under light, the supplementation of riboflavin, lumichrome, or lumiflavin caused significant headspace oxygen depletion. The headspace oxygen in sample bottles under light for 72 h was 13.9%, 15.5%, and 15.7%, respectively, for soymilk supplemented with 100 µM of riboflavin, lumichrome, or lumiflavin. Without the supplementation of the flavins, soymilk under light showed a lesser amount of headspace oxygen depletion. The headspace oxygen in soymilk was 19.1%. Among the flavin derivatives studied here, riboflavin was the most efficient at depleting the headspace oxygen in soymilk.
When compared to the samples stored in dark, the headspace oxygen depletion remained higher in soymilk with no flavin supplementation and stored under light. This oxygen depletion may have been caused by the endogenous riboflavin (~3 ppm) in the soymilk used for this study.

A recent study suggests that chemicals should exhibit several specific properties in order to qualify as effective photosensitizers (DeRosa and Crutchley 2002). Some of these specific properties include: a) a high absorption coefficient in the spectral region of the excitation light, and b) a high quantum yield of the triplet state and long triplet state lifetimes. Riboflavin, as well as its degradation products lumichrome and lumiflavin, has a very high absorption coefficient in the near UV wavelength region. Therefore, all three are likely candidates to act as photosensitizers.

Effect of riboflavin, lumiflavin, and lumichrome on the headspace volatile compounds of soymilk

When soymilk was supplemented with riboflavin and stored under light, the total volatile and beany flavor compounds (e.g., hexanal) increased significantly. Singlet oxygen may be involved in the flavor changes of soymilk supplemented with riboflavin (Huang and others 2004a). This study further compares the effect of riboflavin, lumiflavin, and lumichrome on the total volatiles of soymilk under light or in the dark (Figure 35). When soymilk (with or without the supplementation of riboflavin, lumiflavin, or lumichrome) was stored in the dark for up to 72 h, the change of total headspace volatile in those samples was less than twofold. When stored under light,
however, soymilk showed significant changes in total headspace volatile compounds. The total volatile was about seven times the initial volatile concentration after being stored under light for 72 h. When soymilk supplemented with 100 µM riboflavin, lumiflavin, and lumichrome was stored under light, the change of the total headspace volatile was even more significant. After 72 h, the final total headspace volatile was 30-, 20-, and 17-fold of the initial levels. These results support the hypothesis that riboflavin and its photodegradation products, lumiflavin and lumichrome, act as the photosensitizer to cause more significant flavor changes of soymilk under light. Figure 35 shows that riboflavin is more effective than lumiflavin or lumichrome in causing the headspace total volatile changes. This is consistent with the finding from the headspace oxygen depletion analysis.

The singlet oxygen producing capacity of lumichrome in water has been examined under UVA irradiation by other researchers (Joshi 1989). Lumichrome has been implicated as playing an important role in the photodegradation of polyamidehydroxyurethane polymers in aqueous solution (Onu and others 1998). It has also been shown that lumichrome and other alloxazines were efficient photosensitizers for singlet oxygen (Sikorska and others 1998). Riboflavin was depleted and lumichrome appeared after 1 h of light exposure. Riboflavin may have served as the main sensitizer in the first hour for the degradation of atrazine; subsequently, lumichrome served as the main sensitizer (Cui and others 2002).
Effect of ascorbic acid on the formation of headspace volatiles in soymilk supplemented with riboflavin, lumiflavin, or lumichrome

Research has found that singlet oxygen is involved in the flavor deterioration of soymilk (Huang and others 2004a) and whole fat cow milk (Lee 2002) when the samples are supplemented with riboflavin and stored under light. Ascorbic acid is an excellent singlet oxygen and excited triplet sensitizer quencher (Min and Boff 2002) and has been used widely as an antioxidant in food formulations. Inclusion of ascorbic acid in soymilk stored under light inhibits the formation of hexanal and total volatile compounds in a dose dependent manner (Huang and others 2004a). This study tested the effect of ascorbic acid on volatile compound changes of soymilk supplemented with riboflavin, lumichrome, or lumiflavin under light. Table 14 illustrates the inhibitory effect of ascorbic acid on the total volatile formation in soymilk supplemented with riboflavin, lumichrome, or lumiflavin under light. The addition of 50 mM ascorbic acid in soymilk significantly inhibited the formation of the total volatile compounds in soymilk headspace. After 72 h under light at 4°C, the total volatile in soymilk headspace with 50 mM ascorbic acid was only 15% of those containing no ascorbic acid (Table 14). These results suggest that the riboflavin degradation products, lumichrome and lumiflavin, have photosensitization properties similar to riboflavin. Under light storage conditions, riboflavin in soymilk was photodegraded to lumichrome and lumiflavin. In the meantime, all three acted as photosensitizers to form singlet oxygen. The singlet oxygen formed can react with the lipid components in soymilk to cause flavor deteriorations of soymilk (Figure 36).
5.5 Conclusions

Riboflavin is not stable under light and can be quickly degraded. Lumichrome and lumiflavin were identified as the major photodegradation products from riboflavin, depending on the pH of the solution. The degradation products lumichrome and lumiflavin act as photosensitizers, which is similar to riboflavin. They generate singlet oxygen when exposed to light in an aqueous food system. All three had significant effects on the depletion of headspace oxygen and the formation of total headspace volatiles in soymilk under light. Ascorbic acid minimizes the formation of total volatiles in soymilk containing riboflavin, lumiflavin, or lumichrome under light. Most importantly, soymilk should be stored in dark conditions to minimize the possible flavor changes.

5.6 References


Haag WR, Mill T. 1987. Rate constants for interaction of singlet oxygen ($^1D_g$) with azide ion in water. Photochem Photobiol 45:317-21.


Lee JH. 2002. Photooxidation and photosensitized oxidation of linoleic acid, milk and lard. [Ph.D. Dissertation]. Ohio State University, Columbus, OH, USA.


| Under light (h) | pH=4.5  |   |   | pH=6.5  |   |   | pH=8.5  |   |   |
|               | RF  | LF | LC | RF  | LF | LC | RF  | LF | LC |
| 0             | 97.3 | -- | -- | 101.4 | -- | -- | 99.3 | -- | -- |
| 2             | 48.1 | -- | 47.3 | 35.8 | -- | 48.8 | 3.7 | 30.9 | 73.1 |
| 4             | 11.2 | -- | 73.9 | 3.7 | -- | 56.4 | -- | 15.1 | 63.8 |
| 6             | --  | -- | 79.1 | --  | -- | 58.7 | --  | 9.9  | 52.7 |
| 8             | --  | -- | 79.9 | --  | -- | 58.1 | --  | 5.6  | 53.0 |
| 16            | --  | -- | 78.2 | --  | -- | 56.9 | --  | --  | 41.7 |
| 24            | --  | -- | 72.3 | --  | -- | 50.7 | --  | --  | 31.5 |
| 48            | --  | -- | 57.3 | --  | -- | 40.8 | --  | --  | 23.5 |
| 72            | --  | -- | 31.7 | --  | -- | 30.1 | --  | --  | 9.9  |

- **= not detectable.

- Numbers (µM) were the average of duplicated determinations and were calculated from the respective calibration lines.

Table 11  Effect of pH on the change of riboflavin, lumiflavin and lumichrome under light storage.
<table>
<thead>
<tr>
<th>Storage time (h)</th>
<th>RF (µM)</th>
<th>LF (µM)</th>
<th>LC (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 mM NaN₃</td>
<td>5 mM NaN₃</td>
<td>0 mM NaN₃</td>
</tr>
<tr>
<td>0</td>
<td>49.9</td>
<td>50.1</td>
<td>50.2</td>
</tr>
<tr>
<td>2</td>
<td>28.8</td>
<td>43.5</td>
<td>42.3</td>
</tr>
<tr>
<td>4</td>
<td>7.1</td>
<td>38.1</td>
<td>28.3</td>
</tr>
<tr>
<td>8</td>
<td>0.2</td>
<td>28.1</td>
<td>13.5</td>
</tr>
<tr>
<td>24</td>
<td>0.3</td>
<td>10.7</td>
<td>0.4</td>
</tr>
</tbody>
</table>

- Numbers (µM) were the average of duplicated determinations and were calculated from the respective calibration curves.

Table 12  Effects of sodium azide on the retention of 50 µM riboflavin, lumiflavin, or lumichrome under light
Table 13  The slopes and intercepts from the regression lines in Figure 33.

<table>
<thead>
<tr>
<th></th>
<th>Slope (S) (1/mM)</th>
<th>Intercept (mM)</th>
<th>S/I (mM)</th>
<th>k_r (x 10^8 M^-1s^-1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RF</td>
<td>1.0619</td>
<td>4.005</td>
<td>0.2651</td>
<td>9.66</td>
</tr>
<tr>
<td>LF</td>
<td>1.7109</td>
<td>5.732</td>
<td>0.2985</td>
<td>8.58</td>
</tr>
<tr>
<td>LC</td>
<td>3.4665</td>
<td>11.115</td>
<td>0.3119</td>
<td>8.21</td>
</tr>
</tbody>
</table>
The concentration of riboflavin, lumiflavin, and lumichrome was 100 µM.

Volatile compounds are expressed in electronic counts of GC peaks. Numbers are the averages from SPME/GC analysis for duplicated samples. A numbers with * indicates it as significantly different from that of treatment without adding ascorbic acid at the same storage condition.

### Table 14  Effects of ascorbic acid on total volatile compounds of soymilk supplemented with riboflavin, lumichrome, or lumiflavin and stored under light.

<table>
<thead>
<tr>
<th>Storage time (h)</th>
<th>Soymilk + RF</th>
<th>Soymilk + LF</th>
<th>Soymilk + LC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 mM AA</td>
<td>50 mM AA</td>
<td>0 mM AA</td>
</tr>
<tr>
<td>0</td>
<td>288</td>
<td>268</td>
<td>322</td>
</tr>
<tr>
<td>24</td>
<td>1715</td>
<td>675*</td>
<td>1003</td>
</tr>
<tr>
<td>48</td>
<td>6235</td>
<td>922*</td>
<td>2378</td>
</tr>
<tr>
<td>72</td>
<td>9925</td>
<td>1538*</td>
<td>6008</td>
</tr>
</tbody>
</table>
Figure 29  Chromatograms from HPLC.  Top panel: riboflavin (100 µM) under light for 8 h.  Center panel: riboflavin stored under light for 16 h.  Bottom panel: lumichrome standard.
Figure 30 Spectra comparison of riboflavin degradation product (top panel) and authentic lumichrome (bottom panel).
Figure 31  HPLC/MS of the major photodegraded product from riboflavin.
Figure 32  The formation of lumichrome and lumiflavin from riboflavin at different pHs.
Figure 33  Reciprocal plot of the degradation of riboflavin, lumiflavin, and lumichrome under light at different initial concentrations.
Figure 34  Effect of riboflavin, lumiflavin, and lumichrome on the headspace oxygen (%) of soymilk under light or in dark.
Figure 35  Effect of riboflavin, lumiflavin, and lumichrome on the total volatiles of soymilk under light or in dark.
Figure 36 Riboflavin, lumiflavin, and lumichrome as photosensitizers for $^1\text{O}_2$ formation in soymilk and the reaction of $^1\text{O}_2$ with riboflavin, lumiflavin, and lumichrome.
CONCLUSIONS

1. Riboflavin produces singlet oxygen from triplet oxygen under light. The reaction rate between riboflavin and singlet oxygen is $1.01 \times 10^{10} \text{ M}^{-1} \text{s}^{-1}$. This rate is one of the fastest known chemical reaction rates.

2. Ascorbic acid and sodium azide protect riboflavin from photodegradation under light by quenching singlet oxygen and the excited triplet sensitizer. Ascorbic acid quenched both the singlet oxygen and the excited triplet riboflavin under light, while sodium azide quenched only the singlet oxygen.

3. The singlet oxygen quenching rate of sodium azide was $1.547 \times 10^{7} \text{ M}^{-1} \text{s}^{-1}$.

4. Riboflavin degradation under light involved both the Type-I and Type-II mechanisms.

5. The relative contributions of the Type-I and Type-II mechanisms in the riboflavin photosensitized degradation was 25% and 75%, respectively.

6. Lumichrome was identified to be the major photodegradation product from riboflavin at both a neutral and acidic pH.

7. At a basic pH, lumiflavin was also present as one of the major photodegradation products from riboflavin.
8. The reaction rate between lumichrome with singlet oxygen and lumiflavin with
singlet oxygen is $8.6 \times 10^8 \text{ M}^{-1}\text{s}^{-1}$ and $8.2 \times 10^8 \text{ M}^{-1}\text{s}^{-1}$, respectively.

9. Singlet oxygen quencher alone could not completely protect the riboflavin from
degradation under light. Ascorbic acid, which is a quencher of both the singlet
oxygen and the excited triplet riboflavin, can effectively protect riboflavin in
foods during storage under light.

10. Riboflavin had significant effects on the depletion of headspace oxygen and the
formation of total volatile compounds in soymilk under light.

11. Similar to riboflavin, its major photodegradation products lumichrome and
lumiflavin function as effective photosensitizers in foods.

12. Hexanal, which is associated with a beany flavor, is formed by singlet oxygen
oxidation of linoleic acid in soymilk.

13. Fresh soymilk with no beany flavor can produce beany flavor by the singlet
oxygen oxidation in soymilk during storage under light.

14. Ascorbic acid can inhibit the formation of hexanal and total volatile in soymilk
under light.

15. Soymilk should be stored in the dark and could be fortified with ascorbic acid to
minimize the formation of the undesirable volatile compounds such as beany
flavor compounds.
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