INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps. Each original is also photographed in one exposure and is included in reduced form at the back of the book.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.
Riboflavin photosensitized singlet oxygen oxidation in milk products

Bradley, Dondeena G., Ph.D.
The Ohio State University, 1991
RIBOFLAVIN PHOTOSENSITIZED SINGLET OXYGEN OXIDATION IN MILK PRODUCTS

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in the Graduate School of The Ohio State University

By

Dondeena G. Bradley, B. S., M. S.

The Ohio State University
1991

Dissertation Committee:
Dr. Kenneth Lee
Dr. Michael Mangino
Dr. David B. Min
Dr. Andrew Proctor

Approved By
Advisor
Graduate Studies in
Food Science & Nutrition
To my parents
I would like to thank Dr. David B. Min, my advisor, for his diligence in developing my research and writing skills and his insight in preparing me for a career in both industry and academics. A sincere thanks go to my advisory committee, Drs. Ken Lee, Mike Mangino, and Andy Proctor for their time commitment, suggestions and helpful comments. A special thanks to Drs. Takeato Ogata, Dore Meinholdt, and Lawrence Berliner for their expertise and time spent in using electron spin resonance spectroscopy. To my parents, James and Judy, for providing an exceptional home environment for pursuing excellence in every aspect of my life. To my fiance, Allen, who has endured with me during the pursuit of this degree, I've appreciated your endless patience and especially thank you for providing 'comic relief' in bulk - you're the best.
VITA

December 28, 1964...............................Born - Anderson, Indiana
1987.............................................B. S., Anderson College , Anderson, IN
1987 - 1989.......................................M. S., Purdue University, W. Lafayette, IN

PUBLICATIONS


FIELDS OF STUDY

Major Field: Food Science and Nutrition
# TABLE OF CONTENTS

ACKNOWLEDGEMENTS.........................................................................................................................iii
VITA..........................................................................................................................................................iv
LIST OF TABLES..........................................................................................................................................vii
LIST OF FIGURES.......................................................................................................................................viii
INTRODUCTION.........................................................................................................................................1

## II. LITERATURE REVIEW ....................................................................................................................4

1. Oxidation of Milk..................................................................................................................................4

2. Light-Induced Oxidation of Milk.........................................................................................................6

3. Photochemical Processes....................................................................................................................7

4. Mechanism of Photosensitized Oxidation...........................................................................................9
   A. Chemistry of triplet and singlet oxygen.........................................................................................12
   B. Type I and II reaction pathways.....................................................................................................16
   C. Role of riboflavin in photosensitized oxidation of milk...............................................................19

5. Singlet Oxygen Oxidation of Milk......................................................................................................21
   A. Kinetics of singlet oxygen oxidation..............................................................................................22
   B. Quenching mechanism of singlet oxygen oxidation.....................................................................23
   C. Detection of singlet oxygen...........................................................................................................27

6. Electron Spin Resonance Spectroscopy............................................................................................30
   A. Interaction of singlet oxygen with TMPD to form TAN...............................................................33

## III. OBJECTIVES ..................................................................................................................................35

## IV. EXPERIMENTAL PROCEDURES ........................................................................................................36

1. Detection of Singlet Oxygen by ESR Spectroscopy.........................................................................36
   A. Materials..........................................................................................................................................36
   B. Sample preparation of riboflavin solutions and milk samples.....................................................36
   C. Instrument conditions.......................................................................................................................38
   D. Fluorometric method for riboflavin determination in milk...........................................................38

2. Effects, Quenching Mechanisms and Kinetics of Water Soluble Compounds in Riboflavin Sensitized Photooxidation of Milk......................................................................................39
## LIST OF TABLES

<table>
<thead>
<tr>
<th>TABLE</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Flavors and associated compounds in oxidized milk fat</td>
<td>6</td>
</tr>
<tr>
<td>2. The lifetime of singlet oxygen in solution</td>
<td>18</td>
</tr>
<tr>
<td>3. Depleted headspace oxygen (µmol of O₂/mL of headspace) of milkfat samples (M) containing 0.01, 0.03, 0.05 M DABCO and DMF with 40 µM added riboflavin during three hours of illumination</td>
<td>61</td>
</tr>
<tr>
<td>4. Parameters in the plot of [AO₂]⁻¹ vs. [milk fat]⁻¹ to determine the effects of DABCO and DMF on the headspace oxygen depletion of milk containing 40 µM riboflavin under light storage for three hours</td>
<td>65</td>
</tr>
<tr>
<td>5. The fatty acids and their percentages found in milk</td>
<td>97</td>
</tr>
</tbody>
</table>
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>FIGURE</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>9</td>
</tr>
<tr>
<td>2.</td>
<td>10</td>
</tr>
<tr>
<td>3.</td>
<td>11</td>
</tr>
<tr>
<td>4.</td>
<td>13</td>
</tr>
<tr>
<td>5.</td>
<td>15</td>
</tr>
<tr>
<td>6.</td>
<td>22</td>
</tr>
<tr>
<td>7.</td>
<td>24</td>
</tr>
<tr>
<td>8.</td>
<td>25</td>
</tr>
<tr>
<td>9.</td>
<td>26</td>
</tr>
<tr>
<td>10.</td>
<td>34</td>
</tr>
<tr>
<td>11.</td>
<td>43</td>
</tr>
<tr>
<td>12.</td>
<td>45</td>
</tr>
</tbody>
</table>

1. Excitation and deactivation of sensitizer

2. Jablonski's diagram of the excited states of sensitizers

3. The Type I and II pathways of photosensitized oxidation

4. Molecular orbital of triplet oxygen

5. Molecular orbital of singlet oxygen

6. Reaction pathways of singlet oxygen

7. Scheme for the quenching of singlet and triplet oxygen

8. Plot of $[A_0]^2$ vs. $[A]^{-1}$ at various $[Q]$ gives constant y-intercept equal to $K^{-1}$

9. Plot of $S_Q/y$-intercept vs $[Q]$ with y-intercept equal to $k_d/k_r$ and slope of $(k_{ox-Q} + k_q)/k_r$

10. A standard ESR spectrum of TAN

11. ESR spectra of a 50 mM phosphate buffer solution of pH 7.0 containing 5 µM riboflavin and 20 mM TMPD during illumination with oxygen bubbling. The ESR settings were identical in both spectra

12. Effect of illumination on TAN formation in a 50 mM phosphate buffer solution pH 7.0 containing 5 µM riboflavin and 20 mM TMPD and skim milk containing 20 mM TMPD with oxygen bubbling
13. ESR spectra of whole (top) and raw milk (bottom) containing 20 mM TMPD during illumination with oxygen bubbling. The ESR settings were identical in both spectra .......................................................... 46

14. Effects of illumination period on TAN concentration in skim milk containing added 10 µM TAN with oxygen bubbling ............................................................... 48

15. Effect of 5, 10, and 15 µM riboflavin on TAN formation in phosphate buffer and skim milk containing 20 mM TMPD during 5 minutes of illumination with oxygen bubbling .................................................. 49

16. The effect of added riboflavin (5 µM) after a buffer solution containing 5 µM riboflavin and 20 mM TMPD was illuminated for 40 minutes during oxygen bubbling ................................................................. 50

17. Effect of 15 µM riboflavin on ESR spectra of skim milk with and without TMPD during 20 minutes of illumination with oxygen bubbling. The ESR setting were identical in both spectra .................................................. 52

18. Effect of light intensity as measured by distance of sample from light source on signal intensity of TAN in buffer solution containing 5 µM riboflavin and 20 mM TMPD during oxygen bubbling ........................................ 54

19. The effect of pH of a phosphate buffer solution containing 5 µM riboflavin and 20 mM TMPD on the signal intensity of TAN during oxygen bubbling ................................................................. 56

20. The effect of illumination on the signal intensity of TAN at pH 7.87 and pH 8.24 .................................................................................................................. 57

21. The effect of TMPD concentration in a phosphate buffer solution containing 5 µM riboflavin on the signal intensity of TAN ........................................................................... 59
22. Effect of 1,4-diazabicyclo-[2,2,2]-octane (DABCO) on the headspace oxygen depletion of milk containing 40 μM riboflavin under light storage at room temperature for three hours..........................62
23. Effect of dimethylfuran (DMF) on the headspace oxygen depletion of milk containing 40 μM riboflavin under light storage at room temperature for three hours...........................................................................................63
24. Regression line of $S_Q/S_O$ vs. concentrations of 1,4-diazabicyclo-[2,2,2]-octane (DABCO) in milk [$S_Q$ and $S_O$ are the slopes of the plot $[A_{O_2}]^{-1}$ vs. $[\text{milkfat}]^{-1}$ in the presence and absence of quencher, respectively]........................................................................................................................67
25. Regression line of $S_Q/S_O$ vs. concentrations of dimethylfuran (DMF) in milk [$S_Q$ and $S_O$ are the slopes of the plot $[A_{O_2}]^{-1}$ vs. $[\text{milkfat}]^{-1}$ in the presence and absence of quencher, respectively].................................................................68
26. The lowest hyperfine line of the ESR spectrum of TAN generated in buffer solution of pH 8.24 containing 5 μM riboflavin and 20 mM TMPD during oxygen bubbling at 20, 25, 30, 40, 50, and 60 minutes illumination (not actual size)..........................................................................................................................81
27. The ESR spectra of TAN formed in skim milk containing 0, 5, 10, and 15 μM added riboflavin with 20 mM TMPD after 5 minutes of illumination during oxygen bubbling.........................................................................................82
28. The lowest hyperfine line of a standard ESR spectrum of a 0.2 μM TAN solution after 15 minutes of oxygen bubbling in the dark and 10 minutes of illumination for the determination of the conversion factor from signal intensity measured in millimeters to TAN concentration measured in μM (0.2 μM TAN = 58 mm)........................................83
29. The ESR spectra obtained after 5 minutes of illumination for the comparison of the addition of 10 μM TAN (top) with the formation of TAN in the presence of 20 mM TMPD (bottom) in raw milk samples.

30. The standard ESR spectrum of 1 μM TAN in distilled water after 15 minutes oxygen bubbling in the dark and 5 minutes of illumination used to compare with ESR parameters obtained from spectra in model systems at gain of 5 x 10^4.

31. The standard ESR spectrum of 1 μM TAN in whole milk after oxygen bubbling for 15 minutes and illuminated for 5 minutes used for comparison of ESR parameters obtained from spectra in whole milk samples at gain of 1 x 10^5.

32. The lowest hyperfine lines of the ESR spectra obtained from skim milk containing 1 μM TAN stored in the dark with continuous oxygen bubbling at 2, 5, 10, 15, 20 and 30 minutes.

33. The ESR spectra of TAN formed in 2 % milk containing 20 mM TMPD before illumination after 10 minutes of oxygen bubbling (top) and after 5 minutes of illumination (bottom).

34. ESR spectra of TAN obtained in skim milk before illumination after 10 minutes of oxygen bubbling (top), containing 20 mM TMPD after 5 minutes of illumination (middle) and containing 10 mM TMPD after 5 minutes of illumination (bottom).

35. ESR spectra of TAN generated in whole milk containing 20 mM TMPD at 0 (top), 5 (middle) and 15 minutes of illumination (bottom) with oxygen bubbling.
36. ESR spectra of whole milk samples containing 20 mM TMPD before (top) and after 5 minutes of illumination (bottom) without the presence of oxygen.................................................................91

37. ESR spectra of TAN obtained in buffer solution containing 5 μM riboflavin and 20 mM TMPD before illumination (top), in the presence of oxygen (middle), and without oxygen (bottom).........................................................92

38. ESR spectra obtained in phosphate buffer solution containing 5 μM riboflavin and 20 mM TMPD with oxygen bubbling before illumination (top), with methanol at 10 % (w/v) (middle), and without methanol (bottom)........93

39. ESR spectra obtained in skim milk containing 5 μM riboflavin and 20 mM TMPD with oxygen bubbling before illumination (top), with the addition of 30 units/ml SOD (middle-top), without SOD (middle-bottom) and the addition of heat-denatured SOD (bottom) at gain of 1x 10^4........................................................................................................94

40. ESR spectra of 0.2 μM TAN added to skim milk before nitrogen bubbling (top), after nitrogen bubbling (middle) and after illumination for 3 minutes during nitrogen bubbling (bottom)..............................................95

41. The ESR spectra of 0.2 μM TAN added to phosphate buffer containing 5 μM riboflavin during nitrogen bubbling before illumination (top), after 5 minutes illumination (middle) and after 8 minutes illumination (bottom)........................................................................................................96

xii
I. INTRODUCTION

Photosensitized oxidation of foods is initiated during light absorption by natural sensitizers, such as chlorophyll present in vegetable oils, producing an excited state of the sensitizer. This may follow two major reaction pathways, Type I or Type II (Foote, 1968). Type I is the reaction of the excited triplet sensitizer with food components such as protein or fat to produce free radicals and radical ions. Type II is the reaction of the excited triplet sensitizer with atmospheric triplet oxygen producing singlet oxygen (Foote, 1979). Singlet oxygen can directly react with electron-rich compounds such as double bonds of fatty acids to produce undesirable flavor compounds and possible toxic compounds, as well as destroy essential fatty acids (Frankel et al., 1982). The reaction rate of singlet oxygen with linoleic acid is 1,450 times greater than that of normal triplet oxygen with linoleic acid (Rawls and VanSanten, 1970).

Oxidation of milk components is a major concern of the dairy industry (Parks, 1974). Dairy products are excellent sources of riboflavin with an average content of 0.41 milligrams of riboflavin in one cup of milk (McBean and Speckman, 1988). Since milk contributes 40 to 50% of total riboflavin in the United States (Allen and Parks, 1979), the mechanism of riboflavin sensitized oxidation of milk should be carefully examined. It is widely known that milk exposed to light energy within the visible spectrum leads to the development of off-flavor (Aurand et al., 1964; Hoskin and Dimick, 1979; Bradley, 1980; Hedrick and Glass, 1975). Fluorescent light commonly used to illuminate dairy display cases provide optimal conditions for the promotion of deteriorative reactions initiated by riboflavin (Smith and MacLeod, 1955; Sattar and deMan, 1975; Nelson and Cathcart, 1984). Photochemical reactions
of riboflavin and various flavin compounds have been studied in several systems (Penzer, 1970; Growdowski et al., 1977; Fritz, et al., 1987). Riboflavin has been shown to favor a Type I reaction because it is easily oxidized or reduced and its high water solubility minimizes its interaction with oxygen which is more soluble in lipids (Korycka-Dahl and Richardson, 1978). Riboflavin has been suggested to generate singlet oxygen in milk during exposure to light because a singlet oxygen trapper, 1,3-diphenylisobenzofuran and a singlet oxygen quencher, 1,4-diazabicyclo-[2,2,2]-octane (DABCO), decreased the oxidation of milk as measured by TBA values during 5 days of storage (Aurand et al., 1977). However, the formation and detection of singlet oxygen during riboflavin photosensitized oxidation of milk has not been determined by electron spin resonance (ESR) spectroscopy.

The detection of singlet oxygen during photosensitized oxidation is difficult to measure in that its lifetime ranges from nano- to microseconds. ESR spectroscopy has been used to indirectly detect the formation of singlet oxygen in the presence of myoglobin derivatives using a type of spin trapping technique (Whang and Peng, 1988). The compound 2,2,6,6-tetramethyl-4-piperidone (TMPD) can react with singlet oxygen to form a stable nitroxide radical, 2,2,6,6-tetramethyl-4-piperidone-1-oxyl (TAN) which is easily detected by ESR (Lion et al., 1976; Moan and Wold, 1979). However, the method was used in a model system and not a complete food system. The purpose of this experiment was to detect the presence of singlet oxygen during riboflavin photosensitized oxidation of milk using ESR spectroscopy in a whole food such as milk.

The effects of lipid soluble quenchers on minimizing singlet oxygen oxidation in vegetable oils have been extensively studied (Fakourelis et al., 1987; Warner and Frankel, 1987; Lee and Min, 1988; Min and Lee, 1988). However, studies on the
effects of water soluble quenchers on minimizing singlet oxygen oxidation in foods such as milk are limited. Although the water soluble quenchers, 1,3-diphenylisobenzofuran and 1,4-diazabicyclo-[2,2,2]-octane (DABCO) minimized oxidation of milk during exposure to visible light (Aurand et al., 1977), the effects, quenching mechanisms, and kinetics of water soluble quenchers in milk have not been reported.

Further objectives of this research were to determine the effects of the water soluble quenchers, dimethylfuran and DABCO, on the photosensitized oxidation of milk and to determine the quenching mechanisms and kinetics of these compounds during riboflavin photosensitized oxidation of milk.
II. LITERATURE REVIEW

1. OXIDATION OF MILK

Oxidation of milk can lead to rancidity, off-flavor and deterioration of texture. Almost every constituent of milk has been suspected responsible for off-flavor development (Sattar and deMan, 1975), therefore, the knowledge of the chemical composition of milk is essential to study oxidation of milk and milk products. The average composition includes 87.5% water, 3.9% fat, 3.4% protein, 4.8% lactose and 0.8% as minerals (Henderson, 1971). Minor components include chlorides, phosphates, citrates, bicarbonates of sodium, potassium, calcium and magnesium. Although more than 100 components have been identified, the composition varies in response to physiological and environmental factors. Variations in composition occur among different breeds of cow, between milkings and between milk taken from different sections of the udder. Composition is also affected by feed type, environmental temperature, season, and age of cow.

The fat contained in milk is most susceptible to compositional changes influenced by environmental factors such as the type of diet consumed by the animal. The fat in milk primarily exists as triglycerides containing approximately 12.5% glycerol and 85.5% fatty acids by weight. Over 400 different fatty acids have been identified of which 60 to 70% are saturated, 25 to 35% are monounsaturated, and 4% are polyunsaturated (McCarthy et al., 1960). Palmitic, stearic, and oleic acids are the principal fatty acids present in milk. Milk fat is present as individual globules that contain a layer of protein and phospholipids which prevent globules from
combining to form larger fat masses (McCarthy et al., 1960). These phospholipids contain polyunsaturated fatty acid residues which are susceptible to oxidation. The fat globule membrane is surrounded with trace minerals and other substances that accelerate oxidation (McCarthy et al., 1960). The fat globule membrane is a major factor in fat oxidation, therefore, agitation, foaming, temperature cycling and homogenization which alter its composition affect rate of oxidation.

The major proteins present in milk are casein and whey. Casein, 80% of milk protein, can be subdivided into \(\alpha_1\), \(\alpha_2\), \(\beta\), \(\kappa\), and \(\gamma\) caseins. Whey proteins constitute 20% of total milk protein and includes \(\beta\)-lactoglobulins and \(\alpha\)-lactalbumin. Proteases and lipases are mostly responsible for the flavor of milk, but other milk enzymes such as xanthine oxidase, sulfhydryl oxidase, lactoperoxidase, catalase, protease, \(\alpha\)-amylase, \(\beta\)-amylase, and aldolase may play a role in the development of off-flavors. Specifically, the amino acids cystine, methionine, tryptophan, and histidine of casein as well as lactalbumin contribute to off-flavor. Milk enzymes have also been implicated in the development of oxidized flavors (Sattar and deMan, 1975).

The oxidation of milk is influenced by many factors such as oxygen concentration, temperature, pH, presence of catalysts, light, and inhibitors, therefore, the mechanism of oxidation in milk is quite complex. The presence of trace amounts of copper or contamination with iron can catalyze the formation of oxidized flavor (Mulder et al., 1964). In order to reduce oxidative defects, metal contamination is minimized by using materials made of stainless steel or glass during the handling of milk. The replacement of trapped oxygen in the headspace of high fat dairy products with \(\text{CO}_2\) or nitrogen is highly recommended for products stored for long periods at room temperature.
2. LIGHT-INDUCED OXIDATION OF MILK

Light induced off-flavors is common and may be responsible for decreasing milk sales (Sattar and deMan, 1975). Although light can penetrate milk to depths of 18 mm at 5460 Å and 4.5 mm at 3650 Å, light induced chemical changes are not restricted to a few millimeters in liquid foods due to movement during transit and handling. Light induced off-flavors have been described by several names depending on the associated chemical compound as shown in Table 1.

Table 1. Flavors and associated compounds in oxidized milk fat

<table>
<thead>
<tr>
<th>FLAVOR</th>
<th>COMPOUND</th>
</tr>
</thead>
<tbody>
<tr>
<td>oxidized</td>
<td>oct-1-ene-3-one, octanal</td>
</tr>
<tr>
<td>cardboard</td>
<td>n-octanol, n-alkanals (C₂-C₉)</td>
</tr>
<tr>
<td>oily</td>
<td>hex-2-enal</td>
</tr>
<tr>
<td>painty</td>
<td>n-alkanals (C₅-C₁₀)</td>
</tr>
<tr>
<td>grassy</td>
<td>alk-2-enal</td>
</tr>
<tr>
<td>mushroom</td>
<td>oct-1-ene-3-one</td>
</tr>
<tr>
<td>creamy</td>
<td>4-cis-heptenal</td>
</tr>
</tbody>
</table>

(Korycka-Dahl and Richardson, 1978)

The two major types of light induced off-flavors are the activated and oxidized flavors. The activated flavor is described by terms such as burnt, scorched, or mushroom resulting from oxidation of proteins (Sattar and deMan, 1975). The oxidized flavor is described by terms such as cardboard, metallic, or oily caused by oxidation of unsaturated fatty acids (Velander and Patton, 1955). The combination of these flavors have been described as sunlight flavor.
Studies on the oxidation of milk fat exposed to sunlight have shown decreases in oleic and linoleic acid contents of low density lipoproteins implicating them as major substrates for photooxidation reactions (Finley, 1968). It has been reported that photooxidation of methyl linoleate produces high levels of the less stable 11-hydroperoxide (Kahn et al., 1954), which readily form alk-2-enals, the carbonyls found in milk after exposure to sunlight (Wishner, 1964).

The intensity of light and length of exposure are significant factors in the production of off-flavors associated with oxidized milk. Providing effective protection against sunlight or artificial light is recommended for improved shelf-life of milk and milk products. Most milk is packaged in plastic containers which transmits more light than fiberboard containers (Nelson and Cathcart, 1984). Milk stored in plastic containers contained the light-induced flavor over 10 times as frequently as milk packaged in paper containers due to excess light exposure (Reif et al., 1983). Use of pigmented plastic containers, yellow fluorescent lamps or white fluorescent lamps with yellow shields which reduce exposure to damaging light wavelengths were suggested for reduction of light-induced changes in milk (Hansen et al., 1975).

3. PHOTOCHEMICAL PROCESSES

Light consists of electromagnetic radiation and discrete particles existing as an energy unit of distinct magnitude. The relationship between kinetic energy and frequency allows the energy content of light photons to vary inversely with wavelength. The shorter the light wavelength, the higher the energy content of a photon of light exerting a greater effect on chemical reactions. This relationship between wavelength and energy content can be observed in equation 1:
\[ \varepsilon = h \left( \frac{c}{\lambda} \right) \]  

where, \( \varepsilon = \) energy of photon (ergs)  
\( h = \) Planck's constant \( (6.63 \times 10^{-27} \text{ erg-sec}) \)  
\( c = \) velocity of light \( (3.0 \times 10^{10} \text{ cm/sec}) \)  
\( \lambda = \) wavelength (cm)

Visible light is electromagnetic radiation that ranges between 4000-7000 Å. The wavelength of UV light ranges between 2000-4000 Å. Absorption of light energy produces an activated molecule which may undergo several secondary reactions including degradation to heat, collision resulting in energy transfer, formation of new bonds or dissociations into free radicals resulting in chain reactions.

Photosensitized oxidation is a type of light induced reaction occurring in food during exposure to visible light. In this reaction the light absorbing species does not undergo permanent chemical change as shown in equations 2-4:

\[ S + h\nu \rightarrow S^* \]  
\[ S^* + A \rightarrow A^* + S \]  
\[ A^* + B \rightarrow AB \]

where \( S = \) sensitizer and \( A \) and \( B = \) acceptor compounds. Photosensitized oxidation is initiated by sensitizers such as dyes (eosin, rose bengal, crystal violet, acridine orange, methylene blue), pigments (chlorophyll, porphyrins, flavins), and aromatic hydrocarbons (anthracenes, rubrene) (Korycka-Dahl and Richardson, 1978).

Pigments present in food, such as chlorophyll found in vegetable oils, are efficient photosensitizers due to their conjugated double bond system which easily absorbs visible light energy (Usuki et al., 1984).
4. **MECHANISM OF PHOTOSENSITIZED OXIDATION OF MILK**

The interaction of light, photosensitizers and oxygen is the basis for the formation of singlet oxygen. This potentially damaging mechanism begins with the absorption of light by photosensitizers where light absorption is dependent upon the arrangement of electrons around the atomic nuclei in the photosensitizer. The rates of excitation and deactivation of a sensitizer during the absorption of light energy is shown in Figure 1.

![Figure 1. Excitation and deactivation of sensitizer.](image)

As light energy is absorbed by the sensitizer, an electron is boosted to a higher energy level and the sensitizer is referred to as an unstable singlet excited (\( ^1\text{Sen}^* \)) state. Only a few picoseconds are required for a sensitizer molecule to absorb energy and be transformed into a singlet excited state. When light energy is removed, the electrons rapidly lose energy while returning to the lower energy ground state. The excited
singlet state can then undergo intersystem crossing (ISC) to the triplet excited state of the sensitizer ($^3\text{Sen}^*$) which can participate in photooxidation or gradually decay to the ground state of the sensitizer. The lifetime of the excited triplet sensitizer is approximately 20,000 times longer than the lifetime of the singlet state of the sensitizer. The singlet excited state of the sensitizer may undergo three physical processes: Internal conversion, emission of light, or intersystem crossing as illustrated by Jablonski's diagram (REF) in Figure 2.

![Jablonski's diagram of singlet and triplet states of photosensitizers.](image)

Figure 2. Jablonski's diagram of singlet and triplet states of photosensitizers.

Internal conversion is the transformation of one excited state into another of the same spin state resulting in loss of energy as heat. Not only can energy be lost as heat, but the singlet excited sensitizer may emit light on decay to the ground state. Since the emission originates and terminates in the same multiplicity state, the emission is
called fluorescence. Excitation of molecules by light and their fluorescent decays are extremely fast processes. The excited singlet may also be converted to a triplet excited state via intersystem crossing. The excited state of the triplet sensitizer goes to a lower triplet energy state which may decay to the ground state of the singlet state by emitting light. Since the emission originates from the triplet state and terminates in a singlet state, the emission is called phosphorescence.

Photosensitizers have two excited states, the singlet and the triplet. The sensitizer absorbs ultraviolet (200 - 400 nm) or visible (400 - 700 nm) light and is converted to an excited singlet sensitizer (1Sen*) via absorption of a photon. The short-lived excited 1Sen* undergoes intersystem crossing (ISC) to the long-lived excited triplet sensitizer (3Sen*). It is the triplet excited state of the sensitizer which is the reactive intermediate in photosensitized oxidation. 3Sen* can follow two reaction pathways (Type I and Type II) as shown in Figure 3.

\[
\begin{align*}
\text{Scn} \rightarrow_{hv}^{\text{hv}} 1\text{Scn}^* \rightarrow & \text{ISC} \rightarrow 3\text{Scn}^* \\
+ \text{RH} & \rightarrow \text{R} + \text{Scn H}^+ \\
+ 3\text{O}_2 & \rightarrow \text{ROOH} \\
\end{align*}
\]

\[
\begin{align*}
&k_1 + 3\text{O}_2 \\
&\rightarrow \text{ROOH} \\
&k_2 + 3\text{O}_2 \\
&\rightarrow \text{O}_2^- + \text{Scn}^+ \\
&\text{ROOH} \\
&+ \text{RH} \\
&\rightarrow \text{ROOH} \\
\end{align*}
\]

Figure 3. The Type I and II pathways of photosensitized oxidation.
Type I is the reaction of the excited triplet sensitizer with substrate or solvent to produce free radicals and radical ions. These radicals can react with oxygen to produce oxygenated products. Type II is the reaction of the excited triplet sensitizer with atmospheric triplet oxygen to produce singlet state oxygen. Once singlet oxygen is formed it initiates oxidation by reacting with electron dense compounds forming hydroperoxides that readily break down to produce undesirable compounds (Frankel et al., 1982; Neff et al., 1983; Terao and Matsushita, 1977). In order to facilitate the discussion of singlet oxygen oxidation of milk as well as quenching and kinetic reactions of singlet oxygen, a simplified description of the chemistry of ground state and excited molecular oxygen is included.

A. Chemistry of triplet and singlet oxygen

The discussion of the mechanism of photosensitized oxidation must include the chemical nature of oxygen and its activated states. Although molecular oxygen has an even number of electrons, the highest energy orbitals contain a pair of doubly degenerate antibonding orbitals with only two electrons (Kearns, 1971). The molecular orbital theory best describes the electron structure of molecular oxygen and its excited states (Korycka-Dahl and Richardson, 1978). Molecular oxygen, which consists of two oxygen atoms, has ten molecular orbitals containing twelve valence electrons. Valence electrons are added sequentially to the orbitals in order of increasing energy to obtain the molecular orbital diagram of triplet oxygen as shown in Figure 4. According to Pauli's exclusion principle only two electrons can occupy each orbital. Hund's rule states that one electron is placed into each orbital of equal energy one at a time before the addition of the second electron. Pauli's exclusion principle also states that electrons in a given orbital must have opposite spins. An electron in an atom or molecule generates two
Figure 4. Molecular orbital of triplet oxygen.
types of magnetic momenta and mechanical angular momenta. This behavior occurs from the motion of the electron around the nucleus and from the "spin" of the electron. Electrons of opposite spin are assigned to each orbital. If the resultant spin (S) of the ground state of the molecule is zero then the multiplicity of the state as determined by the equation, $2S + 1$, is said to be one. An excited state is formed by removing one of the electrons from the upper most filled orbital (bonding $\pi$) of the ground state to a vacant orbital (antibonding $\pi^*$) of higher energy. The ground state of most stable molecules containing an even number of electrons is diamagnetic because of the arrangement of the electrons into pairs with opposed spin magnetic moments. The ground state for most molecules is singlet until a molecule is excited to the triplet state and is paramagnetic by virtue of the total spin magnetic moment of the two unpaired electrons. Oxygen, however, is an exception to this generality. The two highest energy electrons of triplet oxygen illustrate the diradical nature of oxygen with an unpaired electron in each of the two highest degenerate orbitals.

Singlet oxygen, whose electrons are paired, is a violation of Hund's rule creating an electronic repulsion that can produce five excited states. Two of the most common include an activated $^1\Sigma$ state which lies 37.5 kcal above the ground state and an activated $^1\Delta$ state occurring 22.4 kcal above the ground state. The $^1\Sigma$ state of oxygen has two electrons with opposite spins in different orbitals and is so reactive that it is not able to survive relaxation to the ground state. The less energetic $^1\Delta$ state of molecular oxygen is sufficiently stable long enough to react with other singlet state molecules and is shown in Figure 5. The $^1\Delta$ state is responsible for most singlet oxygen reactions, therefore, singlet oxygen is subsequently used to designate $^1\Delta$ state of oxygen (Foote, 1968).
Figure 5. Molecular orbital of singlet oxygen.
Singlet oxygen is suggested to be responsible for initiating lipid oxidation because of its low energy of only 22.4 kcal above the ground state, its relatively long lifetime (several microseconds), and its highly electrophilic nature seeking electrons from electron rich compounds to occupy its vacant molecular orbital (Rawls and VanSanten, 1970). Once this active species is formed it is responsible for initiating oxidation which rapidly produces free radicals that in turn can initiate a free radical chain reaction. This species can readily react with double bonds of fatty acids or other compounds which contain high densities of electrons. Relative reactivity rates of singlet oxygen have shown that arachidonic acid has the highest reactivity followed by linolenate, linoleate, and oleate, with ratios of 3.5: 2.9: 1.9: 1.1, respectively (Doleiden et al., 1974). Relative reactivity ratios of fatty acids such as linolenate, linoleate and oleate with triplet oxygen were 25:12:1, respectively (Sonntag, 1979).

Singlet oxygen can be generated chemically (Khan and Martell, 1967; Rawls and VanSanten, 1968; Waters, 1970; Rosenthal, 1985), enzymatically (McCord and Fridovich, 1968), by gaseous discharge (Bader and Orgryzlo, 1964; Wayne, 1969), and by decomposition of hydroperoxides (Cahill and Taube, 1952). Its formation by photochemical methods is most important in food systems whose natural photosensitizers can generate singlet oxygen (Carlsson et al., 1972; Clements et al., 1973; Foote and Denny, 1968). The following is a detailed discussion of Type I and II pathways of photosensitized oxidation and their relationship to foods.

B. Type I and II pathways

The Type I pathway is characterized by hydrogen atom or electron transfer between an excited triplet sensitizer and a reducing substrate resulting in the production of free radicals (Livingston, 1961). The excited triplet sensitizer serves
as a photochemically activated free radical initiator which reacts with triplet oxygen to
produce oxidized compounds that readily break down to form free radicals. The rate of
the Type I reaction depends on the type and concentration of sensitizer and substrate.
For example, the sensitizer benzophenone abstracts hydrogen from a simple alcohol
10,000 times faster than eosin, however, eosin and benzophenone react at similar
rates with more powerful reductants like N,N-dimethylaniline (Foote, 1976). It is
generally accepted that readily oxidizable or reducible compounds favor this
sensitizer-substrate pathway. High substrate reactivity and concentration and low
oxygen concentration favor the Type I reaction.

The Type II pathway is an energy transfer process between an excited triplet
sensitizer with triplet oxygen via triplet-triplet annihilation to generate singlet
oxygen. This fast reaction accounts for almost all of the transfer of energy from the
excited triplet sensitizer to triplet oxygen. Less than one percent of triplet oxygen is
converted to superoxide ion and an oxidized form of the sensitizer by electron transfer
from the triplet sensitizer to triplet oxygen. Electron-rich compounds such as olefins,
dienes, and aromatic compounds favor the Type II pathway. The rate of the Type II
reaction depends on the solubility and concentration of oxygen present in the food.
Oxygen is more soluble in lipids and nonpolar solvents than in water, therefore, traces
of chlorophyll in vegetable oils would tend to promote photosensitized oxidation by the
Type II pathway (Samuel and Steckel, 1974). Foods containing high concentrations of
water such as milk would tend to favor sensitizer-substrate reactions due to the
inaccessibility of oxygen to interact with the excited triplet sensitizer.

The competition between substrate and triplet oxygen for the excited triplet
sensitizer is a major determinant for a Type I or Type II reaction. The type of reaction
may change during the course of photosensitized oxidation as the concentration of
substrate and oxygen changes. The decay rate of singlet oxygen to the ground state and the rate at which it reacts with a particular substrate must also be considered. Reactions of singlet oxygen with unsaturated fatty acids is dependent on the lifetime of singlet oxygen in a solvent. Kinetic spectroscopic analysis of the disappearance of an oxidizable substrate following pulse generation of singlet oxygen has been a widely used technique for the determination of singlet oxygen lifetimes (Adams and Wilkinson, 1972; Kearns, 1979; Monroe, 1985). The lifetime of singlet oxygen depends on the type of solvent present as shown in Table 2 (Min et al., 1989).

Table 2. The lifetime of singlet oxygen in solution.

<table>
<thead>
<tr>
<th>SOLVENT</th>
<th>LIFETIME (μsec.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protiated Acetone</td>
<td>46.5 ± 2.0</td>
</tr>
<tr>
<td>Deuterated Acetone</td>
<td>690 ± 20</td>
</tr>
<tr>
<td>Protiated Acetonitrile</td>
<td>54.4 ± 1.3</td>
</tr>
<tr>
<td>Deuterated Acetonitrile</td>
<td>600 ± 33</td>
</tr>
<tr>
<td>Protiated Benzene</td>
<td>26.7 ± 1.3</td>
</tr>
<tr>
<td>Deuterated Benzene</td>
<td>550 ± 11</td>
</tr>
<tr>
<td>Protiated Water</td>
<td>4.0</td>
</tr>
<tr>
<td>Deuterated Water</td>
<td>68.1 ± 2.5</td>
</tr>
</tbody>
</table>

For instance, the lifetime of singlet oxygen is 4 μsec in water (Rodgers and Snowden, 1982) as compared to 25-100 μsec in nonpolar solvents. In aqueous-lipid systems, the longer half-life of singlet oxygen in the lipid phase favors oxidation of compounds that partition into the lipids. Temperature has little effect on the reaction rate of singlet oxygen oxidation as indicated by negligible changes in the lifetime of singlet
oxygen at a temperature range of -37.6 to 21.6° C (Ogilby and Foote, 1983). This suggests that the lifetime of singlet oxygen in food systems is largely dependent on the type of matrix, water or fat, present in the food product. Milk, for instance, is 85% water whereas vegetable oils are nearly a 100% nonpolar matrix. Understanding these pathways are extremely important in milk that contains substantial amounts of riboflavin which can undergo photocatalyzed reactions (Jenness, 1959).

C. Role of riboflavin in photosensitized oxidation of milk

Riboflavin is a constituent of flavoproteins naturally found in milk which is capable of absorbing light implicating its participation as a photosensitizer during photosensitized oxidation of milk. Milk exposed to visible or UV light leads to the development of off-flavor (Aurand et al., 1964; Hoskin and Dimick, 1979; Bradley, 1980; Hedrick and Glass, 1975). Riboflavin can break down easily during exposure to light. The major product of photodegradation of riboflavin in milk exposed to sunlight was identified as 7,8-dimethylalloxane (Parks and Allen, 1977). The decomposition reaction rate was dependent on the milk composition: 1.86 x 10^{-5}/sec in skim milk and 1.47 x 10^{-5}/sec in whole milk and oxygen was found to protect riboflavin from photolysis (Allen and Parks, 1979). The loss of riboflavin was as high as 30% during a 30 minute exposure of milk in a room using fluorescent lamps (Wishner, 1964).

Not only can the absorption of light by riboflavin lead to the development of off-flavors and photodegradation, riboflavin can initiate photosensitized oxidation of light by absorbing light energy producing an excited triplet state of riboflavin that follows the Type I or Type II pathway (Aurand et al., 1977). Riboflavin, which is readily oxidized or reduced, would tend to follow the Type I pathway although it has also been suggested to participate via a singlet oxygen route (Korycka-Dahl and Richardson,
The formation of singlet oxygen in milk was suggested based on the inhibitory effects of a singlet oxygen trapping agent such as 1,3-diphenylisobenzofuran (DPBF) or a physical singlet oxygen quencher, 1,4-diazabicyclo-[2,2,2]-octane (DABCO) on lipid oxidation. Data, however, were collected during five days of storage under illumination whereas photosensitized oxidation normally begins within several hours after light absorption by the sensitizer initiating a free radical chain reaction. Thus, the role of the triplet excited state of riboflavin during photosensitized oxidation of milk fat has not been defined.

The properties and behavior of riboflavin in its excited-state may be different from the ground state molecule (Cairns and Metzler, 1971). The lowest singlet excited state of riboflavin is of ($\pi, \pi^*$) character corresponding to the 445 nm absorption band. The higher singlet excited state ($n, \pi^*$) states are related to the photochemical reactivities of riboflavin. Intersystem crossing from this state to the triplet excited state is very efficient in aqueous solutions at room temperature. Four $n, \pi^*$ states are possible in riboflavin due to the presence of two pyridine-like nitrogens and carbonyl oxygens and are important for describing the photochemical characteristics of riboflavin.

Electronic excitation energy at the upper excited singlet state in riboflavin can undergo internal conversion to the lowest singlet state or undergo intersystem crossing to the nearby triplet state, however, some competition may occur between the two processes. Although fluorescence emission in riboflavin is quite efficient, phosphorescence intensity from riboflavin is said to be rather weak (Fritz et al., 1987). Competition may occur between the rate of radiationless transition from the triplet to the ground state and the rate of energy transfer from triplet state of riboflavin to oxygen.
In contrast to the photochemistry in the singlet excited state of riboflavin, the major route of the triplet-state photochemistry appears to be intramolecular photoreduction of the isoalloxane ring of the ribityl side chain. Analysis of the photoreduced reaction mixture shows many products (Fritz et al., 1987). Water as the hydrogen donor for the photoreduction of riboflavin has been ruled out. The intramolecular hydrogen abstraction in the triplet flavin has been described by the molecular orbital method and hydrogen to be abstracted should be coplanar with the ring (Fritz et al., 1987). During light absorption, riboflavin is reduced and able to transfer electrons to suitable hydrogen acceptors. The existence of an intermediate, unstable semiquinone was demonstrated during photooxidation of riboflavin (Sattar and deMan, 1975).

5. SINGLET OXYGEN OXIDATION OF MILK

Although lipid oxidation of milk is caused by a free radical mechanism, the formation of hydroperoxides (ROOH) via direct interaction between molecular triplet oxygen and unsaturated fatty acids (RH) is not likely. This reaction would require a change in total spin in that RH and ROOH are in singlet states while oxygen is in the triplet state (\(^3\text{O}_2\)). These reactions are forbidden because spin is not conserved. Spin conservation would be satisfied if singlet oxygen (\(^1\text{O}_2\)) replaced \(^3\text{O}_2\) as the active species. A role of \(^1\text{O}_2\) oxidation at the initiation step of lipid oxidation has been suggested in that \(^1\text{O}_2\) can react directly with olefinic compounds (Rawls et al., 1970).
A. Kinetics of singlet oxygen oxidation

There are three major reaction pathways available to the highly reactive singlet oxygen molecule as shown in Figure 6.

![Figure 6. Reaction pathways of singlet oxygen.](image)

Path I demonstrates the natural decay of singlet oxygen to ground state triplet oxygen. Singlet oxygen is quenched (A) in Path II and the reaction of singlet oxygen with an acceptor (A) to produce oxidized product is shown by Path III. The depletion rate equation of singlet oxygen and an acceptor (A) to produce oxidized product (AO₂) is shown in equation 5:

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

(5)

where $K$ is the rate of singlet oxygen formation.
During the reaction of singlet oxygen with olefins, Paths I and III are competitive inhibition pathways in that quenching of singlet oxygen by olefins is not expected (Manring et al., 1983). Thus, \( k_q[A] << k_d \) and the equation becomes:

\[
\frac{d[AO_2]}{dt} = \frac{k_r[A]}{k_r[A] + k_d} = \frac{[A]}{[A] + \beta}
\]  

The acceptor half-value concentration \( \beta \) defined as \( k_d/k_r \) is the concentration of acceptor \( A \) at which half of singlet oxygen reacts with acceptor \( A \) to produce oxidized product \( AO_2 \). The rate of decay \( k_d \) is solvent dependent and does not change, thus the \( k_r \) value is used to measure reaction rates of different systems.

B. Quenching mechanism of singlet oxygen oxidation.

Quenching of singlet oxygen includes both chemical and physical quenching (Foote, 1979). Chemical quenching is the reaction of singlet oxygen with a quencher \( Q \) to produce oxidized products \( QO_2 \). Physical quenching is that interaction which leads only to the deactivation of singlet oxygen to triplet oxygen with no oxygen consumption or product formation.

The schematic diagram for the formation of oxidized product \( AO_2 \) via singlet oxygen oxidation is shown in Figure 7 (Foote, 1979). This diagram shows two kinds of quenching in singlet oxygen lipid oxidation: 1) singlet oxygen quenching and 2) triplet sensitizer quenching.
Negligible singlet sensitizer quenching due to its short lifetime gives the steady state kinetic equation as derived from Figure 7 for oxidized products (AO₂) is expressed in equation 7:

\[
\frac{d[AO_2]}{dt} = K \left( \frac{k_o[O_2]}{k_o[O_2] + k_Q[Q]} \right) \times \left( \frac{k_f[A]}{k_f[A] + (k_{ox-Q} + k_q)[Q] + k_d} \right) \tag{7}
\]

where, \( K = \) rate of triplet sensitizer formation.

In the case where there is only singlet oxygen quenching \((k_Q[Q] \ll k_o[O_2])\), equation 7 becomes:

\[
\frac{d[AO_2]}{dt} = K \left( \frac{k_f[A]}{k_f[A] + (k_{ox-Q} + k_q)[Q] + k_d} \right) \tag{8}
\]

where \( K = \) the rate of formation of singlet oxygen.
Equation 8 can be rewritten as:

\[
\frac{d[AO_2]}{dt}^{-1} = K^{-1} \left\{ 1 + [(k_{ox-Q}+k_q) [Q] + k_d/k_r(A)] \right\} \tag{9}
\]

The plot of \(\frac{d[AO_2]}{dt}^{-1}\) vs. \([A]^{-1}\) at various \([Q]\) gives constant y-intercept equal to \(K^{-1}\) as shown in Figure 8.

![Figure 8](image)

Figure 8. Plot of \(\frac{d[AO_2]}{dt}^{-1}\) vs. \([A]^{-1}\) at various \([Q]\) gives constant y-intercept equal to \(K^{-1}\)

With quencher, the slope of this plot \((S_Q)\) is \(K^{-1} \{(k_{ox-Q} [Q] + k_q [Q] + k_d)/k_r\}\).

Without quencher, the slope \((S_O)\) is \(K^{-1} (k_d/k_r)\). The ratio of the slope with quencher \((S_Q)\) to y-intercept gives \((k_{ox-Q} [Q] + k_q [Q] + k_d)/k_r\) and thus \([Q]\) dependent. The plot of \(S_Q/y\)-intercept vs. \([Q]\) has another y-intercept equal to \(k_d/k_r\) and slope of \((k_{ox-Q} + k_q)/k_r\) as shown in Figure 9. If the decay rate of singlet oxygen is known, the reaction rate of substrate with singlet oxygen and the total singlet oxygen quenching rate can be determined. Because \(S_Q/S_O\) is \(\{(k_{ox-Q} [Q] + k_q [Q]/k_d) + 1\}\), the plot of \(S_Q/S_O\) vs. \([Q]\) gives a straight line whose slope is \((k_{ox-Q} + k_q / k_d)\), from which the rate constant of total quenching \((k_{ox-Q} + k_q)\) can also be measured when \(k_d\) is known.
Figure 9. Plot of $S_Q/y$-intercept vs. $[Q]$ has another $y$-intercept equal to $k_d/k_r$ and slope of $(k_{ox-Q} + k_q)/k_r$.

In the case where there is only triplet sensitizer, $(k_{ox-Q} + k_q)$, $[Q] \ll k_r [A] + k_d$, the reaction equation becomes as follows:

$$
\frac{d[A02]}{dt} = K \frac{k_o [O_2]}{k_o [O_2] + K_Q [Q]} \times \frac{k_r [A]}{k_r [A] + k_d}
$$

(10)

where $K$ = the rate of triplet sensitizer formation.

The plot of $(d [AO_2] / dt)^{-1}$ vs. $[A]^{-1}$ at various $[Q]$ gives $y$-intercept equal to $K^{-1}(1 + k_Q [Q]/ k_o [O_2])$. The slope of this plot is equal to $K^{-1} (k_d (k_Q [Q] + k_o [O_2]))/k_o [O_2] k_r$. The ratio of slope to the $y$-intercept of this plot is $k_d/k_r$ and independent of $[Q]$.
C. Detection of singlet oxygen

The detection of singlet oxygen in any reaction is difficult because of its short lifetime, however, several methods have been developed including chemical traps, quenchers, and chemiluminescence (Foote, 1976; Krinsky, 1977). Singlet oxygen oxidation products are different from those produced in a free radical chain reaction. The structural pattern of photosensitized oxidized products can determine the existence of singlet oxygen. For instance, autoxidation of linoleic acid produces only conjugated hydroperoxides whereas singlet oxygen oxidation can produce nonconjugated hydroperoxides and conjugated products.

A method of characterizing singlet oxygen as a reaction intermediate is to study the effect of replacing water with deuterium oxide (Foote, 1985). The lifetime of singlet oxygen is 10 - 20 times longer in deuterium oxide (D\textsubscript{2}O) than water (H\textsubscript{2}O), therefore, singlet oxygen reactions would be more easily detected. The difference between pH and pD, effect of H-D exchange on the active sites of the system under study, rates of free radical reactions in D\textsubscript{2}O verses H\textsubscript{2}O, excited state lifetimes and intersystem crossing efficiencies in D\textsubscript{2}O verses H\textsubscript{2}O must be considered in relation to the deuterium effect. Simply observing a change in the rate of a reaction in D\textsubscript{2}O is not a specific test verifying the existence of singlet oxygen. The method does have several limitations that must be considered before characterizing singlet oxygen as a reaction intermediate (Foote et al., 1984). For example, the lifetime of singlet oxygen must be limited by solvent for the isotope effect to be observed. If all the singlet oxygen is being scavenged by substrate, no effect will be observed.

Chemical traps are often used, but the product must be specific for singlet oxygen if the method is to be useful. Furans were one of the first types of compounds to be used as traps (Foote, 1985). They are oxidized to the ketone as the final product via
an intermediate endoperoxide. Other compounds include N, N, N', N'-tetramethylethylene-diamine (TMEDA), and 1,3-diphenylisobenzofuran (DPBF). DPBF absorbs light in the 410 to 440 nm region suggesting a potential role as a photosensitizer (Foote, 1968). The reaction of singlet oxygen with cholesterol gives a characteristic product, 5-α-hydroperoxy-cholesterol whereas other oxidants and radicals do not produce this compound (Kulig and Smith, 1973). Cholesterol reacts only with a fraction of singlet oxygen decreasing the sensitivity of the reaction.

Chemiluminescence has also been extensively used for detecting singlet oxygen in various reactions. Chemiluminescence is produced after a chemical reaction yields an electronically excited species that emits light as it returns to its ground state (Khan and Kasha, 1970). The method measures very weak luminescence-like emission given off by molecules excited by reaction with singlet oxygen. A direct assay for singlet oxygen measures chemiluminescence resulting from energy liberated when singlet oxygen decays to the ground triplet state. Two types of singlet oxygen luminescence, the direct emission from a single molecule at 1.27 μm and dimol luminescence at 634 and 704 nm, are most commonly observed. Both these luminescence mechanisms are very inefficient and the infrared emission is very difficult to detect. There are many species that are capable of low level chemiluminescence; if the specific wavelengths of singlet oxygen emission are not observed, visible chemiluminescence is meaningless (Denecke and Krinsky, 1977). The weak emission from singlet oxygen is usually accompanied by light of other wavelengths in complex systems. Although these emissions are assigned to higher vibrational states of singlet oxygen, it seems likely that the extraneous emission comes from excited carbonyls or other species present. Precise wavelength determination is essential for the detection of singlet oxygen.
One of the most powerful techniques for demonstrating the intermediacy of singlet oxygen is by competitive inhibition with a known singlet oxygen acceptor which is not a good Type I substrate. To minimize undesirable singlet oxygen oxidation in lipid foods, the effects of naturally occurring tocopherols and carotenoids on singlet oxygen oxidation have been extensively studied (Matsushita and Terao, 1980; Fakourelis et al., 1987; Min et al., 1989; Lee and Min, 1990). Inhibition of singlet oxygen reactions by other quenchers such as tertiary amines, histidine, DABCO and azide have been useful in the detection of singlet oxygen (Manring et al. 1983; Krinsky, 1977). Amines are highly specific for the delta singlet state of oxygen with their quenching rate constants ranging from $10^5$ to $10^7$ M$^{-1}$ s$^{-1}$. All singlet oxygen quenchers have low ionization potential. Although quenchers are not specific for singlet oxygen, they physically or chemically interact with singlet oxygen to inhibit oxidation. Calculating inhibition of a singlet oxygen reaction by quantitative analysis best determines the characteristics of a quencher from known rate constants. The concentration which quenches half of the product can be given by the expression (Spikes and Swartz, 1978):

$$[Q] = \frac{k_a [A] + k_d}{k_q}$$  \hspace{1cm} (11)

where, $k_a$ is the rate of singlet oxygen reacting with acceptor, $[A]$ is the concentration of acceptor, $k_d$ is the decay rate of singlet oxygen in the medium, and $k_q$ is the quenching rate of the quencher. DABCO is fairly unreactive in that 0.05 M is needed in aqueous solution to quench half the singlet oxygen formed (Spikes and Swartz, 1978). DABCO is also an efficient scavenger of the hydroxy radical so care must be used in interpreting its effects. The azide ion is much more effective than DABCO but is also a
known scavenger of hydroxyl radicals. Histidine probably reacts first to produce an
endoperoxide which can decompose to a complex mixture of products. Addition of these
compounds should inhibit a reaction dependent on singlet oxygen. If, when added at high
concentration the reaction is not inhibited, singlet oxygen is not required for that
particular reaction. If the reaction is inhibited, this does not prove a definite role for
singlet oxygen since these compounds react with hydroxyl radicals often with a greater
rate constant than reactions with singlet oxygen. In simple models the concentration of
acceptor and quencher can be varied and this relationship will be obeyed. The
quenching of singlet oxygen by carotenoids, tocopherols, and histidine which are
naturally present in foods may be important for minimizing singlet oxygen oxidative
reactions.

7. ELECTRON SPIN RESONANCE SPECTROSCOPY

Electron spin resonance (ESR) spectroscopy is a highly sensitive analytical
method which detects the presence of free radicals. The basic theory of ESR
spectroscopy is that radiation of microwave frequency induces transitions between
magnetic energy levels of electrons with unpaired spins (Ayscough, 1967). The
magnetic energy splitting is caused by a static magnetic field. Unpaired electrons are
present in odd molecules, free radicals, transition metals and are produced after
homolytic fission of a covalent bond during illumination with ultraviolet light or
gamma radiation. Unpaired electrons spin at a faster rate than nuclei thus they have a
stronger magnetic field. The spinning electron behaves like a magnet with its poles
along the axis of rotation and is termed a magnetic moment.

In the absence of an external magnetic field, the free electron may exist in one
of two states, + 1/2 or - 1/2, of equal energy and thus is degenerate. The application
of an external static magnetic field, $H_0$, removes the degeneracy and causes the electron to precess. Two energy levels are established, the lower energy state has the spin magnetic moment aligned in the direction of the magnetic field and corresponds to the quantum number, $M_S = -1/2$. The difference in energy between the two levels is given by

$$\Delta E = h\nu = g \beta_n H_0 \quad (12)$$

where $g$, called the spectroscopic splitting factor has a value which is a function of the electron's environment (a value close to 2 for a free electron); $\beta_n$ is the Bohr magneton, a factor for converting angular momentum to magnetic moment. The SI unit of magnetic field strength is a weber per meter squared, which is named a tesla (T). The conversion to gauss is $10,000 \, G = 1 \, T$. Most ESR measurements in solution are made within a frequency of 9500 MHz and a field strength of 3400 G.

The principal components of the ESR spectrometer are a source of microwave radiation, a microwave bridge which provides the means for applying the microwave power to the sample, a steady magnetic field to provide the magnetic field splitting, a detector to measure the microwave power absorbed from the microwave field and a recorder. (The sample is contained in a cylindrical quartz tube between the poles of the magnet).

In a homogeneous magnetic field an unpaired electron can have many energy states. The number of energy states depend on the relative orientation of the magnetic moments of the unpaired electron, the closely associated nuclear spins, and the applied magnetic field. Since the radical electron is delocalized over the whole molecule the unpaired electron interacts with many nuclei. Nuclei possessing a magnetic moment
may interact and cause a further splitting of the electron resonance line. From the number and intensity distribution of the spectral lines, an ESR spectrum will show how many nuclei interact with the radical electron. The hyperfine coupling constant is the distance between associated peaks of a submultiplet and is measured in gauss. Interaction with a nitrogen nucleus will cause a splitting into three lines of equal intensity.

Hyperfine splitting is independent of the microwave frequency used. This allows one to distinguish a nuclear hyperfine interaction from the effects of differences in spectroscopic splitting factor also known as the g-factor. If the separation of two peaks is observed to vary with the microwave frequency, it follows that they correspond to two transition with different g-factors, not to interaction with the nuclear spin. The g-factor for the unbound electron is equal to 2.002319. The exact value of the g-factor reflects the chemical environment because orbital angular momentum of the electron can have an effect on the angular momentum quantum number. To measure the g-factor for free radicals it is convenient to measure the field separation between the center of the nuclear spectrum and that of a reference substance whose g-value is known.

The ESR spectrum gives a time-averaged view of the geometry of a paramagnetic species. The interpretation of the spectra involves the g-factor, the separation of hyperfine lines, the sample concentration, relaxation times, and the linewidths. If the absorption lines are narrow and the spectrum fully resolved, the actual measurements are straightforward. An ESR spectrum is evaluated by calculating the hyperfine coupling constant and counting the number of spectral lines.
A. The interaction of singlet oxygen with TMPD to form TAN.

Compounds which do not contain an unpaired electron can be studied when they are chemically bonded to a stable free radical (Sargent and Gardy, 1976; Swartz, 1986; Fronczis et al., 1987). This radical produces a sharp and simple ESR spectrum that gives detailed information concerning the molecular environment. Five- or six-membered heterocyclic molecules incorporating a nitroxyl group whose nitrogen atom is bonded to a tertiary carbon are widely used to bond to compounds without unpaired electrons. Since singlet oxygen is not a radical, various amines can interact with singlet oxygen forming nitroxide radicals which are readily detected by ESR. There is a high specificity of amines for singlet oxygen and the existence of a charge transfer complex between the amine and singlet oxygen has been proposed (Lion et al., 1976).

A method was developed in which stable nitroxide radicals were generated by reaction with singlet oxygen. An amine compound such as 2,2,6,6-tetramethyl-4-piperidone (TMPD) interacts with singlet oxygen to form a stable nitroxide radical, 2,2,6,6-tetramethyl-4-piperidone-1-oxyl (known as TAN or TEMPONE) which can be detected by ESR spectroscopy (Whang and Peng, 1988). When photosensitized oxidation of foods occurs in the presence of TMPD, free nitroxide radicals are detected if singlet oxygen is produced. TAN formed between the interaction of singlet oxygen and TMPD has a characteristic ESR spectrum as shown in Figure 10. The three hyperfine lines are a result of the coupling of the unpaired electron with an atom of nitrogen ($I=1$).
Although the effects of myoglobin and its derivatives as photosensitizers in singlet oxygen generation have been studied by this method in a model system (Whang and Peng, 1988), the effects of photosensitizers in complete food systems have not been studied.

A major limitation in radical detection by ESR is the requirement for constant radical concentrations of greater than $10^{-8}$ M and reasonable spectral resolution requires 100- to 1000- fold these levels (Schaich and Borg, 1980). Measurement times vary from tens of seconds to hours, whereas radical lifetimes in solution are very short (<1 msec.), thus steady-state concentrations generally remain less than $10^{-7}$ M. The use of spin-trapping has been developed to overcome this problem by diminishing the rate of disappearance. Substantial development and refinement of this method is yet to come, but the initial demonstration of indirectly detecting singlet oxygen by ESR spectroscopy has been made.
III. OBJECTIVES

1. Detection of singlet oxygen during riboflavin photosensitized oxidation of milk by electron spin resonance spectroscopy.

2. Determine the effects of DABCO and DMF during riboflavin sensitized photooxidation of milk.

3. Determine the quenching mechanisms of DABCO and DMF during riboflavin photosensitized oxidation of milk.

4. Determine the kinetics of singlet oxygen quenching of DABCO and DMF during riboflavin photosensitized oxidation of milk.
IV. EXPERIMENTAL PROCEDURES

1. DETECTION OF SINGLET OXYGEN BY ESR SPECTROSCOPY

A. Materials.

Riboflavin, methanol, 2,2,6,6-tetramethyl-4-piperidone monohydrate (TMPD), and 2,2,6,6-tetramethyl-4-piperidone-1-oxyl (TAN or TEMPONE) were obtained from Aldrich Chemical Company (Milwaukee, WI). Superoxide dismutase (SOD) from bovine erythrocytes was purchased from Sigma Chemical Co. (St. Louis, MO). Whole and skim milk were obtained from a local supermarket.

B. Sample preparation of riboflavin solutions and milk samples.

Distilled water stock solutions of 100 μM riboflavin, 200 mM TMPD, and 50 mM phosphate buffer solution of pH 7.0 were prepared. The 5, 10, and 15 μM riboflavin solutions were prepared from the stock solutions. A 0.05 M phosphate buffer was prepared at pH 7.0 by adding 0.866 g Na₂HPO₄ (0.061 M) and 0.538 g NaH₂PO₄ · H₂O (0.039 M) in a 100 ml of distilled water. The 25 mM buffer solution containing 5, 10, 15 μM riboflavin and 20 mM TMPD were prepared using the stock solutions to maintain the pH of the riboflavin solution close to the pH of milk. The 200 mM TMPD stock solution was added to milk to achieve a final concentration of 20 mM TMPD in milk.

To study the effects of illumination on TAN in riboflavin solution and skim milk, 10 ml of 5 μM riboflavin solution having 20 mM TMPD or skim milk containing 20
mM TMPD was stored under fluorescent light (ca 4,000 lux) for 0, 5, 10, 15, 20, 25, 30, and 40 minutes.

To study the effects of illumination on added TAN in skim milk, the skim milk containing added 10 \( \mu \text{M} \) TAN was stored under fluorescent light or dark for a period of 10, 20, 30, and 40 minutes.

To study the effects of riboflavin on TAN formation in buffer solution or skim milk, the phosphate buffer solution containing 5, 10, or 15 \( \mu \text{M} \) riboflavin and 20 mM TMPD and skim milk containing added 5, 10, or 15 \( \mu \text{M} \) riboflavin and 20 mM TMPD were stored for 5 minutes under light.

To study the effects of TMPD on TAN formation in skim milk containing added 15 \( \mu \text{M} \) riboflavin, skim milk containing added 15 \( \mu \text{M} \) riboflavin with and without 20 mM TMPD were stored under light for 20 minutes with oxygen bubbling.

To study the effects of possible hydroxyl radical interaction with TMPD in riboflavin solutions and skim milk, a 10 % methanol (v/v) in 5 \( \mu \text{M} \) riboflavin solution containing 20 mM TMPD and 10 % methanol in milk containing 20 mM TMPD were stored under light for 20 minutes with oxygen bubbling. To study the effects of possible superoxide anion interaction with TMPD in riboflavin solutions and skim milk, 30 units/ml superoxide dismutase (SOD) in 5 \( \mu \text{M} \) riboflavin solution containing 20 mM TMPD and 30 units/ml superoxide dismutase (SOD) in milk containing 20 mM TMPD were stored under light for 20 minutes with oxygen bubbling.

All samples were bubbled with oxygen (Medipure Oxygen USP, Danbury, CT) in a 200 ml beaker at a flow rate of 100 ml/min. Oxygen was bubbled through water to avoid evaporation. The sample beaker was illuminated with a Sylvania fluorescent lamp (F15T8/CW 15 W) at approximately 4,000 lux. An aliquot of 0.1 ml was placed in a thin ESR flat cell for ESR spectrum measurement at ambient temperature.
C. Instrument conditions.

ESR spectra were obtained using a Varian E-109 X-Band ESR Spectrometer (Palo Alto, CA) with microwave power of 20 mW, magnetic field of 3390 ± 50 G, with 100 kHz modulation amplitude of 2 or 4 G.

D. Fluorometric method for riboflavin determination in milk.

A Turner Model 430 Spectro Fluorometer with a xenon lamp power supply (Palo Alto, CA) determined the riboflavin concentration of milk samples. The excitation wavelength was set at 440 nm and the detection wavelength was set at 565 nm. Milk samples were prepared as follows.

The pH of the milk samples were adjusted to 5.0-6.0 with 0.01N hydrochloric acid. Water (20 ml) was added so the total volume of milk was greater than 10 times the dry weight of the samples in grams. Concentrated HCl (0.4 ml) was added and the samples heated for 30 minutes at 121° C. The pH was adjusted with vigorous agitation to pH 6.0 with sodium hydroxide solution. The filtrate was separated from the solution using Whatman’s No. 4 filter paper.

Ten milliliters of the filtrate were placed into three test tubes labeled 1-3. One milliliter of a riboflavin standard (1 μg/ml) was added to tube 1 and 1 ml of water was added to tubes 2 and 3. One milliliter of acetic acid was added to each tube followed by 0.5 ml 4.0% potassium permanganate solution. The samples were allowed to stand for 2 minutes, then 0.5 ml of 3.0% hydrogen peroxide solution was added and samples were shaken vigorously until bubbling was not observed. The fluorescence of milk in tube 1 containing the riboflavin standard (X) and tube 2 were measured (B). Twenty milligrams of sodium thiosulfate were added to tube 3 and the fluorescence was
measured (C). The concentration of riboflavin in milk was determined by the following equation:

\[ \text{mg riboflavin/ml milk} = [(B-C) / (X-B)] \times 0.10 \times 0.001 \]

2. EFFECTS, QUENCHING MECHANISMS AND KINETICS OF WATER SOLUBLE COMPOUNDS IN RIBOFLAVIN SENSITIZED PHOTOOXIDATION OF MILK.

A. Materials.

Milk and cream in fiberboard cartons from a local supermarket, 1,4-diazabicyclo-[2,2,2]-octane (DABCO) from Sigma Chemical Co. (St. Louis, MO), and dimethylfuran (DMF) from Aldrich Chemical Co. (Milwaukee, WI) were used.

B. Sample preparation, storage and analyses for studying the effects of DABCO and DMF on riboflavin photosensitized oxidation of milk.

To study the effects of water soluble singlet oxygen quenchers, 1,4-diazabicyclo-[2,2,2]-octane (DABCO) and dimethylfuran (DMF) on the photosensitized oxidation of milk, samples of 0.01, 0.03, and 0.05 M DABCO and DMF in milk containing 40 μM riboflavin were prepared in duplicate (Lee and Min, 1988).

Thirty milliliters of milk were transferred into a 35 ml serum bottle. The bottles were sealed air-tight with Teflon-coated rubber septa and aluminum seals and exposed to light and dark storage conditions for three hours. Samples were placed in a light chamber consisting of rectangular glass (60 cm x 30 cm x 50 cm) lined on four sides with white paper. Four fluorescent F401F lamps (Sylvania Co., Danver, MA)
were placed below the glass chamber where light intensity at the sample level was 4,000 lux (Lee and Min, 1988). The light chamber was operated at room temperature. Samples were rearranged in the light chamber every hour to optimize uniform light exposure.

The oxidative stability of milk was determined by measuring oxygen disappearance in the headspace of the serum bottles (Mistry and Min, 1987). Forty microliters of headspace of the sample were manually injected into a Hewlett-Packard 5880A gas chromatograph equipped with a thermal conductivity detector using a 1 ml gas tight syringe (Hamilton Co., Reno, NV). A stainless steel column (1.83 m x 0.32 cm i.d.) packed with 80/100 mesh molecular sieve 13x (Alltech Associates, Inc., Deerfield, IL) was used with helium gas at a flow rate of 30 ml/min. The oven, injector port and detector temperatures were 40, 120, and 80 °C, respectively. Gas chromatographic peak area of oxygen in one ml of headspace gas was measured using a Hewlett-Packard 3390 electronic integrater and expressed as electronic counts. The electronic counts of O₂/ml of headspace gas can be converted to μmoles O₂/ml headspace gas. One milliliter of air contains 0.20946 ml of oxygen (Parker, 1982) and 22,400 milliliters of oxygen is equal to 10⁶ μmoles according to Avogadro's Law. Therefore, 0.20946 ml of oxygen is equal to 9.35 μmoles of oxygen, that is one milliliter 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 1 milliliter of air into the gas chromatograph and electronic counts of 1 μmole oxygen was calculated.
C. Sample preparation and analyses for studying the quenching mechanisms and kinetics of DABCO and DMF in milk.

The quenching mechanisms and kinetics of DABCO and DMF in riboflavin-sensitized photooxidation of milk were studied by the steady-state kinetic equation (Foote and Denny, 1968; Yamauchi and Matsushita, 1977; Zweig and Henderson, 1975). Samples containing 0.10, 0.14, 0.21, 0.41 M milkfat with 40 μM added riboflavin and 0.01, 0.03, and 0.05 M DABCO or DMF were prepared in duplicate. One mole of milkfat was calculated to be 803 grams in one liter (Henderson, 1971). Cream containing 36% milkfat was diluted with deionized water to achieve 0.10, 0.14, 0.21, and 0.41 M milkfat. Thirty milliliters of sample were transferred into a 35-ml serum bottle, air-tightly sealed and placed in the light chamber for 3 hours. The quenching mechanisms and kinetics of DABCO and DMF in milk were studied by measuring the depleted headspace oxygen of the sample bottle by gas chromatography (Fiorti, 1977; Fakourelis et al., 1987). Depleted headspace oxygen content was then expressed as micromoles of oxygen per milliliter of headspace gas.

D. Statistical Analyses. Headspace oxygen values reported are mean values of duplicate samples. Tukey's range test (SAS, 1985) was used to determine the effects of different levels of DABCO and DMF on the depleted headspace oxygen of milk samples during storage.
V. RESULTS & DISCUSSION

1. DETECTION OF SINGLET OXYGEN BY ESR SPECTROSCOPY

A. Effects of illumination on TAN formation in riboflavin solution.

The ESR spectrum of a phosphate buffer solution (pH 7.55) of 5 μM riboflavin containing 20 mM TMPD during 10 minutes of illumination at gain 1 x 10^5 during oxygen bubbling is shown in Figure 11. This spectrum was identified as TAN by comparing the hyperfine coupling constant (16.1 G), g-factor (2.0048), and the line width of the ESR spectrum with those of standard 0.5 μM TAN under identical ESR conditions. The ESR signal intensity was correlated with the peak height of the low field line of the three line ESR spectrum. The ESR spectrum of 20 mM TMPD containing no riboflavin was the same as the background spectrum of Figure 11. Therefore, the background spectrum was due to residual TAN contained in the stock solution of TMPD. The content of TAN was determined by comparing the spectrum peak of the solution with that of standard TAN solution and was estimated to be 0.3 ppm. The same sample containing 5 μM riboflavin and 20 mM TMPD stored under the dark did not produce TAN. Similarly, a buffer solution containing TMPD or riboflavin only under light storage did not produce TAN. Therefore, riboflavin and light were required for singlet oxygen formation. Additional evidence of riboflavin involvement in singlet oxygen formation in milk during illumination was obtained from milk treated with Florisil which removes riboflavin (Aurand et al., 1966). No TAN spectrum was observed in Florisil treated skim milk containing 20 mM TMPD after 30 minutes of illumination.
Figure 11. ESR spectra of a 50 mM phosphate buffer solution of pH 7.0 containing 5 μM and 20 mM TMPD during illumination with oxygen bubbling. The ESR settings were identical in both spectra.
B. Effects of illumination on TAN in milk.

The effects of illumination time on TAN formation in skim milk containing 20 mM TMPD as compared to buffer containing 5 μM riboflavin and 20 mM TMPD shown in Figure 12 indicated that singlet oxygen was produced in skim milk. Singlet oxygen was also produced in whole and raw milk containing 20 mM TMPD as observed in the ESR spectra of TAN after illumination as shown in Figure 13. The concentration of TAN increased during the first 10 minutes of illumination and then gradually decreased as the illumination time increased. The decrease of TAN could be attributed to the reduction of TAN to the corresponding hydroxylamine by reduced riboflavin. After the decay of TAN was observed in a 5 μM riboflavin solution containing 20 mM TMPD illuminated for 30 minutes, the sample was reoxygenated. The TAN signal intensity slightly increased suggesting that the hydroxylamine of TAN may be oxidized to form TAN while reoxygenating the sample increasing the TAN signal. The reduction of nitroxides by reduced riboflavin to give the corresponding hydroxylamine has been documented previously (Chan and Bruice, 1977). TAN is reduced through free radical mechanisms during aerobic photoreduction of hematoporphyrin by pyrogallol (Reszka and Sealy, 1984). The decrease of TAN during longer storage under light suggests the ability of photoreacted riboflavin to reduce TAN to the hydroxylamine form of TAN.
Figure 12. Effect of illumination on TAN formation in a 50 mM phosphate buffer solution of pH 7.0 containing 5 µM riboflavin and 20 mM TMPD and skim milk containing 20 mM TMPD with oxygen bubbling.
Figure 13. ESR spectra of whole and raw milk containing 20 mM TMPD during illumination with oxygen bubbling. The ESR settings were identical in both spectra.
C. Effects of illumination on milk containing 10 μM TAN.

The detection of the TAN signal in milk was difficult due to the observance of a decay phenomenon of TAN in milk. The effect of illumination on skim milk containing 10 μM TAN is shown in Figure 14. The concentration of TAN decreased rapidly after 15 minutes of illumination although the concentration of TAN of the sample stored in the dark showed only a very gradual decrease. The decrease in TAN during light exposure may indicate some type of reaction of TAN with either milk components or riboflavin. When 100 μM TAN was added to skim milk, the decay leveled at a TAN concentration of 49 μM after 60 minutes of illumination suggesting exhaustive reactions with riboflavin and/or reducing agents in milk. This behavior was not observed in milk after riboflavin was removed. 50 μM TAN was added to Florisil treated skim milk and TAN decay was not observed after 60 minutes of illumination. This observation suggests that riboflavin is involved in TAN decay in milk under light storage.

D. Effects of riboflavin on TAN formation in buffer solution and skim milk.

The effects of 5, 10, or 15 μM riboflavin in buffer solution and milk containing 20 mM TMPD on the formation of TAN after 5 minutes of illumination are shown in Figure 15. As riboflavin was increased from 5 to 10 μM the TAN signal increased. However, the addition of more than 10 μM riboflavin decreased the intensity of TAN. Riboflavin may be reduced under light and reduced riboflavin might reduce TAN into the hydroxylamine of TAN decreasing the TAN signal. The addition of 5 μM riboflavin to a solution of 20 mM TMPD and 5 μM riboflavin that had been illuminated for 30 minutes causes an increase in the concentration of TAN as observed in Figure 16.
Figure 14. Effects of illumination period on TAN concentration in skim milk containing added 10 μM TAN with oxygen bubbling.
Figure 15. Effects of 5, 10, and 15 μM riboflavin on TAN formation in phosphate buffer and skim milk containing 20 mM TMPD during 5 minutes of illumination with oxygen bubbling.
Figure 16. The effect of added riboflavin (5 μM) after a buffer solution containing 5 μM riboflavin and 20 mM TMPD was illuminated for 40 minutes during oxygen bubbling.
E. Effect of riboflavin on ESR spectra of skim milk with and without TMPD.

The ESR spectra of skim milk containing added 15 \( \mu \)M riboflavin with and without 20 mM TMPD are shown in Figure 17. Skim milk containing added 15 \( \mu \)M riboflavin and no TMPD showed a new broad signal with a g-factor of 2.0034 which is similar to the g factor of 2.0032 for flavoprotein radicals (Wertz and Bolton, 1972). The flavoprotein radical might be formed between the interaction of light and milk flavoproteins by a Type I reaction. Skim milk containing added 15 \( \mu \)M riboflavin and 20 mM TMPD had the same broad signal with a g-factor of 2.0034 and TAN signal. The observation of a flavoprotein radical and TAN in skim milk containing added riboflavin and TMPD may indicate that both Type I and Type II riboflavin reactions were occurring as expected. As the concentration of excited triplet state riboflavin is increased, the Type II reaction may shift towards the reaction of excited triplet state riboflavin with milk components as the availability of oxygen decreases. This shift from Type I to Type II would increase the production of free radicals such as the flavoprotein radical in milk other than TAN.

F. The effect of oxygen on TAN.

After 15 minutes of nitrogen bubbling into a solution of buffer containing 5 \( \mu \)M riboflavin and 20 mM TMPD, an aliquot of the sample was placed in a closed ESR flat cell and illuminated for 5 minutes. The sample did not produce the ESR spectrum of TAN. Since triplet oxygen was not adequately present, the reaction of excited triplet riboflavin to form singlet oxygen by a Type II reaction was not favorable (Foote, 1979).
Figure 17. Effect of 15 μM riboflavin on ESR spectra of skim milk with and without TMPD during 20 minutes of illumination with oxygen bubbling. The ESR settings were identical in both spectra.
G. The effect of light on the signal intensity of TAN.

The effect of light intensity during sample illumination on the concentration of TAN is shown in Figure 18. Light intensity was changed by increasing the distance of the sample from the light source. The signal intensity of TAN decreased 30% when the distance of the photosensitizing solution from the light source was increased from 1.5 cm to 7.0 cm. The opacity of milk is due to its content of suspended particles of fat, proteins, and certain minerals. The color varies from white to yellow according to the coloration of the fat. Skim milk is more transparent with a slight blue tinge.

H. Effects of superoxide dismutase and methanol on TAN formation.

It was suggested that TMPD can react with hydroxyl radicals or superoxide anion which might be present in milk to form TAN (Kono, 1991). Methanol which is a scavenger of hydroxyl radicals (Spikes and Swartz, 1978) was added to 5 μM riboflavin containing 20 mM TMPD, or to skim milk containing 20 mM TMPD to measure its effect on TAN formation. The addition of methanol to either the riboflavin solution or milk did not change the signal intensity of TAN. This suggests that the hydroxyl radical was not responsible for the formation of TAN.

Superoxide dismutase, which converts the superoxide anion into hydrogen peroxide and triplet oxygen, was added to 5 μM riboflavin solution or skim milk containing 20 mM TMPD to detect the possible involvement of superoxide anion with TMPD to form TAN. Both SOD and heat denatured SOD gave a minimal effect on change in signal intensity of TAN. Since SOD proteins can react with singlet oxygen due to the presence of histidine, tryptophan, and methionine residues which react with singlet oxygen, a control using heat-denatured SOD was used (Halliwell and Gutteridge, 1987). The ESR spectrum of TAN was still observed after the addition of SOD to the
Figure 18. Effect of distance of sample from light source on signal intensity of TAN in buffer solution containing 5 μM riboflavin and 20 mM TMPD during oxygen bubbling.
photosensitizing solution eliminating the potential reaction of superoxide anion with TMPD to form TAN. This also suggests that superoxide anion was not responsible for the formation of TAN in skim milk.

I. Effect of pH on the interaction of TMPD with singlet oxygen.

Milk contains buffering substances (phosphates, carbonates, citrates, and proteins) which emit hydrogen ions at the same rate as hydroxide ions are added with the base, neutralizing most of the added base without any significant changes in the pH. After the addition of 20 mM TMPD solution (pH 9.5) to a 5 μM riboflavin solution, the pH was 2.65 units higher than the pH of milk (pH 6.8). Phosphate buffer was added to keep the pH of the photosensitizing solution of riboflavin and TMPD near the pH of milk containing 20 mM TMPD (pH 7.8).

Signal intensity of TAN during illumination of the phosphate buffer solution containing 5 μM riboflavin and 20 mM TMPD was influenced by the pH of the sample solutions. The effect of pH of a phosphate buffer solution containing 5 μM riboflavin and 20 mM TMPD on the signal height intensity of TAN after 5 minutes of illumination is shown in Figure 19. As the pH of the photosensitizing solutions containing 5 mM riboflavin and 20 mM TMPD increased, the signal intensity of TAN increased. The pH of the photosensitizing solution containing riboflavin and TMPD changes the form of TMPD usable for trapping singlet oxygen. The effect of illumination time on the signal intensity of TAN in a buffer solution containing 5 μM riboflavin and 20 mM TMPD at pH 7.87 and pH 8.24 is shown in Figure 20. Although the signal intensity of TAN is higher at pH 8.24 than pH 7.87, the same phenomenon of TAN decay is observed. The use of TMPD as a trapping compound in foods with low pH would not be feasible for the detection of singlet oxygen. The $k_b$ value of TMPD was calculated to be $5.0 \times 10^{-8}$ M
Figure 19. The effect of pH of a phosphate buffer solution containing 5 μM riboflavin and 20 mM TMPD on the signal intensity of TAN during oxygen bubbling.
Figure 20. The effect of illumination time on the signal intensity of TAN at pH 7.87 and pH 8.24.
from the pH of a 20 mM TMPD solution where the pH was 9.50. The higher the pH of
the sample solution, the higher the percent usable form of TMPD. For example, at pH
9.5, 99.84% of TMPD is in the form that can readily react with singlet oxygen,
however, at pH 7.5, only 86% of TMPD can be used. A TAN signal could not be obtained
when TMPD-HCl was used to make the TMPD stock solution. The monohydrate form of
TMPD was used indicating that pH of the stock solution is an important factor for
observing the TAN spectra.

The pH of the photosensitizing solution may also effect the concentration of
TMPD required for the reaction with singlet oxygen to form TAN. The production of
TAN in the buffer solutions and milk samples were linearly dependent on TMPD
concentration as shown in Figure 21. The higher concentration increased the
sensitivity of detection, therefore 20 mM was used in both aqueous and milk solutions.
At 0.5 mM TMPD present in the photosensitizing solution, no signal of TAN could be
detected.
Figure 21. The effect of TMPD concentration in a phosphate buffer solution containing 5 μM riboflavin on the signal intensity of TAN.
2. EFFECTS, QUENCHING MECHANISMS, AND KINETICS OF WATER SOLUBLE COMPOUNDS IN RIBOFLAVIN SENSITIZED PHOTOOXIDATION OF MILK.

A. Effect of DABCO and DMF on riboflavin photosensitized oxidation of milk.

The effects of 0, 0.01, 0.03, and 0.05 M DABCO and DMF on the headspace oxygen depletion of milk samples containing 40 μM added riboflavin during three hours of light storage are shown in Table 3. The average coefficient of variance (n = 5) of headspace oxygen determination by gas chromatography was 1.5 %. Preliminary studies (data not shown) showed that headspace oxygen was not depleted in milk without riboflavin during 3 hours of illumination. Headspace oxygen depletion of samples with 0.41 M milkfat containing 40 μM added riboflavin stored after 3 hours of illumination without water soluble DABCO or DMF averaged 9.17%.

Headspace oxygen depletion of milk containing added riboflavin under light storage was due to the formation of singlet oxygen as a result of the interaction of riboflavin and light. As the concentration of DABCO and DMF increased, the depleted headspace oxygen content decreased (P < 0.05). The presence of 0.01, 0.03, and 0.05 M DABCO and DMF reduced the amount of headspace oxygen depleted as expected (Table 3). DABCO and DMF acted as antioxidants in milk containing riboflavin under light storage by minimizing the formation of singlet oxygen.
Table 3. Depleted headspace oxygen (µmol of O₂/mL of headspace) of milkfat samples (M) containing 0.01, 0.03, 0.05 M DABCO and DMF with 40 µM added riboflavin during three hours of illumination.

<table>
<thead>
<tr>
<th>QUENCHER (M)</th>
<th>Depleted Headspace Oxygen (µmol of O₂/mL of headspace)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.10 M</td>
</tr>
<tr>
<td>DABCO</td>
<td></td>
</tr>
<tr>
<td>0.00</td>
<td>1.79a</td>
</tr>
<tr>
<td>0.01</td>
<td>0.95b</td>
</tr>
<tr>
<td>0.03</td>
<td>0.70c</td>
</tr>
<tr>
<td>0.05</td>
<td>0.52d</td>
</tr>
<tr>
<td>DMF</td>
<td></td>
</tr>
<tr>
<td>0.00</td>
<td>1.79a</td>
</tr>
<tr>
<td>0.01</td>
<td>0.77b</td>
</tr>
<tr>
<td>0.03</td>
<td>0.47c</td>
</tr>
<tr>
<td>0.05</td>
<td>0.34d</td>
</tr>
</tbody>
</table>

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

B. Quenching mechanisms and kinetics of DABCO and DMF during riboflavin photosensitized oxidation of milk.

The effects of 0, 0.01, 0.03 and 0.05 DABCO and DMF on the headspace oxygen depletion of 0.10, 0.14, 0.21, 0.41 M milkfat containing 40 µM added riboflavin during three hours of light storage are shown in Figure 22 and 23, respectively. As the concentrations of DABCO and DMF increased, the depleted headspace oxygen content of the sample bottles decreased. The antioxidant effects of DABCO and DMF in milk containing riboflavin under light storage may be due to the quenching of singlet oxygen and/or riboflavin. If DABCO and DMF reduced riboflavin-photosensitized oxidation of
Figure 22. The effect of 1,4-diazabicyclo-[2,2,2]-octane (DABCO) on headspace oxygen depletion of milk containing 40 μM riboflavin under light storage at room temperature for three hours.
Figure 23. Effect of dimethylfuran (DMF) on the headspace oxygen depletion of milk containing 40 μM riboflavin under light storage at room temperature for three hours.
milk by singlet oxygen quenching, the following steady-state kinetic equation is established (Foote and Denny, 1968):

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

where \( K \) denotes the rate of singlet oxygen formation; \( AO_2 \), oxidized milkfat; \( k_r \), reaction rate constant of milkfat with singlet oxygen; \( A \), milkfat; \( k_q \), reaction rate constant of physical singlet oxygen quenching by DABCO or DMF; \( k_{ox-Q} \), reaction rate constant of chemical singlet oxygen quenching by DABCO or DMF; \( Q \), DABCO or DMF; and \( k_d \), the decay rate of singlet oxygen in a water solvent. The plot of \( [AO_2]_1 \) vs. \( [A]_1 \) at various \( [Q] \) gives constant y-intercept equal to \( K^{-1} \). Constant intercepts of the plots \( [AO_2]_1 \) vs. \( [A]_1 \) at various \( [Q] \) are diagnostic of singlet oxygen quenching (Foote, 1979). In the presence of quencher, the slope of this plot \( S_Q \) is \( K^{-1} \left\{ (k_{ox-Q} [Q] + k_q [Q] + k_d)/k_r \right\} \). Without quencher, the slope \( (S_Q) \) is \( K^{-1} (k_d/k_r) \). The intercepts of the plots are independent of the concentration of quencher and the slopes are dependent on the concentration of quencher (Foote, 1979). The \( k_d \) values are known for many solvents (Hurst et al., 1982). Because \( S_Q/S_O \) is \( \left\{ (k_{ox-Q} [Q] + k_q [Q]/k_d) + 1 \right\} \), the plot of \( S_Q/S_O \) vs. \( [Q] \) gives a straight line whose slope is \( \left\{ (k_{ox-Q} + k_q /k_d) \right\} \), from which the rate constant of total quenching \( (k_{ox-Q} + k_q) \) can also be measured when \( k_d \) is known.

The results of the study of the quenching mechanism of DABCO and DMF during singlet oxygen oxidation of milk are shown in Figure 11 and 12, respectively. The y-intercepts of the plots \( [AO_2]_1 \) vs. \( [A]_1 \) at 0.01, 0.03, 0.05 M of DABCO and DMF are the same but the slopes are different. The steady-state kinetic equation states when y-intercepts of plots \( [AO_2]_1 \) vs. \( [A]_1 \) at different concentrations of quencher are the
same and the slopes of the plots are different, the quencher minimizes singlet oxygen oxidation by quenching singlet oxygen only. Therefore, DABCO and DMF quenched singlet oxygen only and did not quench the excited triplet state of riboflavin to minimize the riboflavin-photosensitized oxidation of milk.

The slopes and the ratios of $S_Q/S_O$ of the plots containing 0, 0.01, 0.03, and 0.05 M DABCO and DMF calculated from Figures 22 and 23 are given in Table 4.

Table 4. Parameters in the plot of $[\text{A}O_2]^{-1}$ vs. $[\text{milk fat}]^{-1}$ to determine the effects of DABCO and DMF on the headspace oxygen depletion of milk containing 40 μM riboflavin under light storage for three hours.

<table>
<thead>
<tr>
<th>QUENCHER</th>
<th>INTERCEPT (ml headspace/μmole $O_2$)</th>
<th>SLOPE (M-ml headspace/μmole $O_2$)</th>
<th>$S_Q/S_O$ a</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DABCO</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.00</td>
<td>0.144</td>
<td>0.04</td>
<td>1.00 M</td>
</tr>
<tr>
<td>0.01</td>
<td>0.164</td>
<td>0.09</td>
<td>2.25 M</td>
</tr>
<tr>
<td>0.03</td>
<td>0.163</td>
<td>0.13</td>
<td>3.25 M</td>
</tr>
<tr>
<td>0.05</td>
<td>0.172</td>
<td>0.18</td>
<td>4.50 M</td>
</tr>
<tr>
<td>DMF</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.00</td>
<td>0.144</td>
<td>0.04</td>
<td>1.00 M</td>
</tr>
<tr>
<td>0.01</td>
<td>0.178</td>
<td>0.11</td>
<td>2.75 M</td>
</tr>
<tr>
<td>0.03</td>
<td>0.151</td>
<td>0.19</td>
<td>4.75 M</td>
</tr>
<tr>
<td>0.05</td>
<td>0.173</td>
<td>0.28</td>
<td>7.00 M</td>
</tr>
</tbody>
</table>

a $S_Q/S_O$ are the slopes of the plot $[\text{A}O_2]^{-1}$ vs. $[\text{milk fat}]^{-1}$ in the presence and absence of DABCO and DMF, respectively.

The slope and y-intercept of the plot $[\text{A}O_2]^{-1}$ vs.$[\text{A}]^{-1}$ without quencher were 0.04 ml of headspace gas/μmol of oxygen and 0.144 ml of headspace gas/μmol of oxygen, respectively. The linear regression line for the plot of $[\text{A}O_2]^{-1}$ vs.$[\text{A}]^{-1}$ without DABCO
is \( y = 0.04 (x) + 0.144 \), where \( y = [A02]^{-1} \) and \( x = [A]^{-1} \). The slope/intercept ratio of the regression line for milk without DABCO is equal to \( k_d/k_r \) where \( k_d \) is the decaying rate of singlet oxygen in water and \( k_r \) is the reaction rate constant of singlet oxygen with milk (Foote, 1979). The \( k_d/k_r \) value was 0.28 M. The \( k_d \) singlet oxygen in water is \( 2.27 \times 10^5 \) sec\(^{-1} \) (Rodgers and Snowden, 1982). Therefore, the reaction rate constant (\( k_r \)) of singlet oxygen with milk was \( 2.27 \times 10^5 \) sec\(^{-1} \)/0.28 M = 8.1 \times 10^5 \) M\(^{-1}\) sec\(^{-1}\) in water.

To determine the total singlet oxygen quenching rate (\( k_{ox-Q}+k_q \)) of DABCO, the regression line of \( S_Q/S_O \) verses [DABCO] was plotted using the data in Table 4 and is shown in Figure 24. \( k_{ox-Q} \) and \( k_q \) are chemical and physical singlet oxygen quenching rate constants of the quencher, respectively (Foote, 1979). \( S_Q \) and \( S_O \) are the slopes of the plot \( [A02]^{-1} \) vs. \([A]^{-1}\) in the presence and absence of quencher, respectively. The slope of the regression line of the plot \( S_Q/S_O \) verses [DABCO] is \( (k_{ox-Q}+k_q)/k_d \) (Foote and Denny, 1968; Yamauchi and Matsushita, 1977; Foote, 1979). The slope of the regression line of the value of \( S_Q/S_O \) verses [DABCO] was 66.10 M\(^{-1}\). Since the decaying rate constant of singlet oxygen in water was \( 2.27 \times 10^5 \) sec\(^{-1}\) the total singlet oxygen quenching rate constant (\( k_{ox-Q}+k_q \)) of DABCO in milk was \( 1.5 \times 10^7 \) M\(^{-1}\) sec\(^{-1}\).

To determine the total singlet oxygen quenching rate (\( k_{ox-Q}+k_q \)) of DMF, the regression line of \( S_Q/S_O \) verses [DMF] was plotted using the data in the Table 4 and is shown in Figure 25. The slope of the regression line of the value of \( S_Q/S_O \) verses [DMF] was 116.10 M\(^{-1}\). Since the decaying rate constant of singlet oxygen in water was \( 2.27 \times 10^5 \) sec\(^{-1}\) the total singlet oxygen quenching rate constant (\( k_{ox-Q}+k_q \)) of DMF in milk was \( 2.6 \times 10^7 \) M\(^{-1}\) sec\(^{-1}\). The total quenching rate of DMF is 1.7 times faster than DABCO in milk.
Figure 24. Regression line of $S_Q$ and $S_O$ vs. concentrations of 1,4-diazabicyclo-[2,2,2]-octane (DABCO) in milk. $S_Q$ and $S_O$ are the slopes of the plot $[A_0]^2$ vs. $[A]^{-1}$ in the presence and absence of quencher, respectively.
$y = 1.2627 + 116.10x \quad R^2 = 0.991$

Figure 25. Regression line of $S_Q$ and $S_O$ vs. concentrations of dimethylfuran (DMF) in milk. $S_Q$ and $S_O$ are the slopes of the plot $[A^{-1}O_2]$ vs. $[A]^{-1}$ in the presence and absence of quencher, respectively.
VI. CONCLUSIONS

1. The presence of singlet oxygen during riboflavin photosensitized oxidation of milk was indirectly detected by electron spin resonance spectroscopy in the presence of 2,2,6,6-tetramethyl-4-piperidone.

2. Light, oxygen, and riboflavin were required for the formation of singlet oxygen in milk.

3. The concentration of TAN decreased in both buffer and milk samples containing 5 μM riboflavin after 10 minutes of illumination suggesting the reduction of TAN to its corresponding amine by photoreduced riboflavin.

4. As the amount of added riboflavin was increased from 5 -10 μM in buffer solution and milk containing TMPD, the concentration of TAN signal increased, however, the addition of more than 10 μM riboflavin decreased the concentration of TAN.

5. Milk with an addition of 15 μM riboflavin showed a broad signal with a g-factor (2.0034) similar to the g-factor (2.0032) for flavoprotein radicals.
6. The presence of a flavoprotein radical and TAN in milk containing added riboflavin and TMPD suggested that Type I and Type II reactions are occurring simultaneously.

7. The higher the pH of the phosphate buffer solution containing 5 μM riboflavin, the higher the signal intensity of TAN.

8. As the concentration of the water soluble quenchers, DABCO and dimethylfuran, were increased from 0 to 0.05 M, headspace oxygen content was maintained minimizing the singlet oxygen oxidation of milk.

9. Steady state kinetic studies of singlet oxygen oxidation of milk showed that the antioxidant activity of DABCO and DMF was due to singlet oxygen quenching and not quenching of the triplet excited state of riboflavin.

10. The singlet oxygen reaction rate constant with milk fat was 8.1 x 10^5 M^-1 sec^-1.

11. Total singlet oxygen quenching rates of DABCO and DMF were 1.5 x 10^7 M^-1 sec^-1 and 2.6 x 10^7 M^-1 sec^-1, respectively.
LIST OF REFERENCES


AOCS. *Official and Tentative Methods*; American Oil Chemists' Society: Campaign, IL, 1980.


Bader, L. W., and E. A. Orgryzlo. 1964. Reactions of oxygen (\(1\Delta_g\)) and oxygen (\(1\Sigma_g\)). *Discuss. Faraday Soc.* 37, 46.


Figure 26. The lowest hyperfine line of the ESR spectrum of TAN generated in buffer solution of pH 8.24 containing 5 μM riboflavin and 20 mM TMPD during oxygen bubbling at 20, 25, 30, 40, 50, and 60 minutes illumination (times shown respectively). The dotted line represents measurement of signal intensity.
Figure 27. The ESR spectra of TAN formed in skim milk containing 0, 5, 10, and 15 μM (shown from top to bottom) added riboflavin with 20 mM TMPD after 5 minutes of illumination during oxygen bubbling.
Figure 28. The lowest hyperfine line of a standard ESR spectrum of a 0.2 µM TAN solution after 15 minutes of oxygen bubbling in the dark and 10 minutes of illumination for the determination of the conversion factor for converting signal intensity measured in millimeters to TAN concentration measured in µM (0.2 µM TAN = 58 mm).
Figure 29. The ESR spectra obtained after 5 minutes of illumination for comparing the addition of 10 μM TAN (top) with the formation of TAN in the presence of 20 mM TMPD (bottom) in raw milk samples.
Figure 30. The standard ESR spectrum of 1 μM TAN prepared in distilled water after 15 minutes oxygen bubbling in the dark and 5 minutes of illumination for comparing the ESR parameters obtained from spectra in model systems at gain of $5 \times 10^4$. 
Figure 31. The standard ESR spectrum of 1 μM TAN in whole milk after oxygen bubbling for 15 minutes and illuminated for 5 minutes for comparing ESR parameters obtained from spectra in whole milk samples at gain of $1 \times 10^5$. 
Figure 32. The lowest hyperfine lines of the ESR spectra obtained from skim milk containing 1 µM TAN stored in the dark with continuous oxygen bubbling at 2, 5, 10, 15, 20 and 30 minutes (shown from left to right).
Figure 33. The ESR spectra of TAN formed in 2% milk containing 20 mM TMPD before illumination after 10 minutes of oxygen bubbling (top) and after 5 minutes of illumination (bottom).
Figure 34. ESR spectra of TAN obtained in skim milk after 10 minutes of oxygen bubbling before illumination (top), containing 20 mM TMPD after 5 minutes of illumination (middle), and containing 10 mM TMPD after 5 minutes of illumination (bottom).
Figure 35. ESR spectra of TAN generated in whole milk containing 20 mM TMPD at 0 (top), 5 (middle), and 15 minutes of illumination (bottom) with oxygen bubbling.
Figure 36. ESR spectra of whole milk samples containing 20 mM TMPD before (top) and after 5 minutes of illumination (bottom) without the presence of oxygen.
Figure 37. ESR spectra of TAN obtained in buffer solution containing 5 μM riboflavin and 20 mM TMPD before illumination (top), in the presence of oxygen (middle), and without oxygen (bottom).
Figure 38. ESR spectra obtained in phosphate buffer solution containing 5 μM riboflavin and 20 mM TMPD with oxygen bubbling before illumination (top), with methanol at 10 % (w/v) (middle), and without methanol (bottom).
Figure 39. ESR spectra obtained in skim milk containing 5 μM riboflavin and 20 mM TMPD with oxygen bubbling before illumination (top), with the addition of 30 units/ml SOD (middle-top), without SOD (middle-bottom), and the addition of heat-denatured SOD (bottom) at gain of 1 x 10^4.
Figure 40. ESR spectra of 0.2 μM TAN added to skim milk before nitrogen bubbling (top), after nitrogen bubbling (middle), and after illumination for 3 minutes during nitrogen bubbling (bottom).
Figure 41. The ESR spectra of 0.2 μM TAN added to phosphate buffer containing 5 μM riboflavin during nitrogen bubbling before illumination (top), after 5 minutes illumination (middle), and after 8 minutes illumination (bottom).
Table 5. The fatty acids and their percentages found in milk.

<table>
<thead>
<tr>
<th>FATTY ACIDS</th>
<th># OF CARBONS</th>
<th>PERCENTAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Butyric</td>
<td>4</td>
<td>3.7</td>
</tr>
<tr>
<td>Caproic</td>
<td>6</td>
<td>2.0</td>
</tr>
<tr>
<td>Caprylic</td>
<td>8</td>
<td>1.3</td>
</tr>
<tr>
<td>Capric</td>
<td>10</td>
<td>2.7</td>
</tr>
<tr>
<td>Lauric</td>
<td>12</td>
<td>4.0</td>
</tr>
<tr>
<td>Myristic</td>
<td>14</td>
<td>7.9</td>
</tr>
<tr>
<td>Palmitic</td>
<td>16</td>
<td>23.8</td>
</tr>
<tr>
<td>Stearic</td>
<td>18</td>
<td>10.7</td>
</tr>
<tr>
<td>Arachidic</td>
<td>20</td>
<td>0.5</td>
</tr>
<tr>
<td>Oleic</td>
<td>18</td>
<td>38.3</td>
</tr>
<tr>
<td>Linoleic</td>
<td>18</td>
<td>4.7</td>
</tr>
</tbody>
</table>

(Henderson, 1971)