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Photoinduced Generation of 2,3-Butanedione from Riboflavin

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The volatile compound formation from riboflavin solution of a phosphate buffer (0.1 M, pH 6.5) under light for 15 h was studied by SPME-GC and SPME-GC/MS analysis. Only one major compound in the riboflavin solution was formed and increased as the light exposure time increased. The light-exposed riboflavin solution had a buttery odor. The compound of riboflavin solution under light was analyzed by gas chromatography and olfactometry. The major volatile compound eluted from the gas chromatograph had a buttery odor. The buttery odor compound was positively identified as 2,3-butanedione by a combination of gas chromatographic retention time, mass spectrum, and odor evaluation of authentic 2,3-butanedione. The addition of sodium azide, a singlet oxygen quencher, to riboflavin solution minimized the formation of the buttery odor compound. Singlet oxygen was involved in the formation of the buttery odor. The 2,3-butanedione was produced from the reaction between riboflavin and singlet oxygen. Singlet oxygen was formed from triplet oxygen by riboflavin photosensitization mechanism. This is the first reported oxidation reaction between riboflavin and singlet or triplet in food and biological systems.

KEYWORDS: Riboflavin; 2,3-butanedione; photosensitized oxidation; singlet oxygen

INTRODUCTION

Riboflavin, vitamin B_2, is an active part of the coenzymes of flavin mononucleotide and flavin adenine dinucleotide, which catalyze many oxidation−reduction reactions in biological systems. These coenzymes play essential roles in several dehydrogenases and oxidases. Riboflavin exists in milk, eggs, meats, vegetables, and many other food products (*1*−*3*). Milk is the most important source of riboflavin in diets in the United States and many other nations. Riboflavin content in whole and skim milk is 1.5−2.0 μg/mL (*4*). Light destroys riboflavin in milk rapidly (*4*−*6*). Riboflavin in foods is extremely unstable under light, but very stable in the dark (*7*, *8*).

Riboflavin has complex photochemical properties (*9*−*11*) and has been extensively studied as a photosensitizer in foods (*4*, *12*−*14*, *21*) and biological systems (*15*, *16*). Riboflavin produced singlet oxygen from ordinary triplet oxygen under light by the excited triplet riboflavin and triplet oxygen annihilation mechanism (*17*). The direct detection of singlet oxygen, which has only about 2 μs of lifetime depending on solvent, was extremely difficult (*18*). The singlet oxygen formed by riboflavin photosensitization was trapped by 2,2,6,6-tetramethyl-4-piperidone and produced stable 2,2,6,6-tetramethyl-4-piperidone-1-oxyl radical. The 2,2,6,6-tetramethyl-4-piperidone-1-oxyl radical was detected by electron spin resonance spectroscopy (*18*). The electron spin resonance spectrum of 2,2,6,6-tetramethyl-4-piperidone-1-oxyl radical formed from riboflavin under light had three hyperfine lines, and the hyperfine coupling constant and *G*-factor were 16.1G and 2.0048, respectively (*18*). The reaction rate between riboflavin and singlet oxygen was 1.01×10^{10} M^{-1} s^{-1} (*7*). This explains the extremely fast degradation of riboflavin in foods under light. Sodium azide reduces the degradation of riboflavin under light by quenching singlet oxygen at the rate of 1.55×10^7 M^{-1} s^{-1} (*7*). The nonvolatile compounds formed from riboflavin under light were lumichrome and lumiflavin (*19*). The lumichrome and lumiflavin were produced from the excited triplet riboflavin by cleaving 3,4,5-trihydroxy-2-pentanone and 2,3,4-trihydroxybutanal, respectively. Singlet oxygen was not involved in the production of lumichrome and lumiflavin (*19*). The reaction products between riboflavin and triplet or singlet oxygen, the identification and chemical mechanisms of the volatile compounds formed from riboflavin, and the flavor qualities of the volatile compounds formed from riboflavin under light have not been reported in the literature. The objectives of this study were to identify the volatile compounds formed from riboflavin under light and to study the chemical mechanisms for the formation of volatile compounds under light and the flavor properties of the volatile compounds from riboflavin.

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MATERIALS AND METHODS

Materials. Riboflavin and sodium azide were purchased from Sigma Chemical Co. (St. Louis, MO). Millipore purification system with a filter of Progard 2 and Milli-Q Plus ultrapure water system with Purification Pak were purchased from Millipore Co. (Bedford, MA). Serum bottles (10 mL), aluminum caps, and Teflon-coated septa were purchased from Supelco, Inc. (Bellefonte, PA). The 15 mm × 1.5 mm magnetic bars were purchased from Bel-Art Products (Pequannock, NJ).

Riboflavin Solution Preparation. Phosphate buffer (0.1 M, pH 6.5) was prepared with the freshly purified water, which was first purified by a Millipore purification system with a filter of Progard and then further purified by a Milli-Q Plus ultrapure water system with Purification Pak. Riboflavin solution (0.5 mM) in the phosphate buffer (0.1 M, pH 6.5) was prepared, and 20 mL of the riboflavin solution was, in triplicate, transferred into a 100-mL Erlenmyer flask. The flask was sealed with Parafilm, and the riboflavin solution was stored in a light storage box of 3000 Lux for 0, 3, 6, 9, 12, and 15 h at 25 °C. The riboflavin solutions stored under light for 0, 3, 6, 9, 12, and 15 h were analyzed for volatile compounds.

Analysis of Headspace Volatile Compounds of Riboflavin Solution by SPME-GC. First, 3 mL of the riboflavin sample solution stored under light or in dark was pipetted into a 15 mL serum bottle having a 15 mm × 1.5 mm magnetic bar. The serum bottle was air-tightly sealed with a Teflon-coated rubber septum and aluminum cap. Headspace volatile compounds of riboflavin serum bottle were analyzed by the SPME method with a 75 μm carboxen/polydimethylsiloxane fiber (Supleco Inc., Bellefonte, PA). The sample serum bottle was kept in a 40 °C water bath for 20 min to equilibrate the sample temperature and to have a good reproducibility for SPME analysis. The SPME fiber was inserted into the headspace of temperature equilibrated riboflavin solution bottle and exposed for 20 min in a 40 °C water bath. The riboflavin solution in the serum bottle was continuously stirred with a 15 mm × 1.5 mm magnetic bar to increase the extraction of headspace compounds and to improve the reproducibility of SPME analysis. The volatile compounds adsorbed in the SPME fiber were desorbed into the injection port of a gas chromatograph (Shimadzu GC-14B, Shimadzu, Japan) for 5 min at 250 °C. The injection port was fitted with a 0.75 mm internal diameter splitless glass liner. Supelco wax 10-fused silica capillary column (60 m × 0.32 mm, 0.25 μm film thickness; Supelco Inc., Bellefonte, PA), a flame ionization detector, and helium gas were used for gas chromatography. The GC oven temperature was held at 40 °C for 2 min, increased to 170 °C at 7 °C/min, and held at 170 °C for 2 min.

Gas Chromatography−Olfactometry. The odor characteristics of the effluents from the gas chromatograph were analyzed by gas chromatography−olfactometry. The effluent from capillary gas chromatographic column was split to a sniffing port by a glass seal Y connector (Supelco Inc., Bellefonte, PA). The riboflavin solution under light for 15 h was used for the gas chromatography−olfactometry analysis. The SPME method was used for the extraction of volatile compounds from the sample headspace as described above. The gas chromatographic conditions and column used for the gas chromatography−olfactometry analysis were the same as those used for SPME-GC analysis.

Gas Chromatography−Mass Spectrometry (GC/MS). The identification of the volatile compounds was carried out with a gas chromatograph−mass spectrometer (Perkin-Elmer). The electron ionization of mass spectra was 70 eV. The gas chromatographic conditions for GC/MS were identical to those used for the SPME-GC analysis.

Effect of Sodium Azide on Volatile Compound Formation of Riboflavin Solution. To study the possible involvement of singlet oxygen in the volatile compound formation of the riboflavin solution, the 0.5 mM riboflavin solutions containing 0, 0.5, 1.0, and 5.0 mM sodium azide were prepared. The riboflavin solutions (20 mL) were transferred into 100-mL Erlenmyer flasks, and the flasks were sealed with Parafilm and stored in the light storage box at 3000 Lux for 12 h.

Effects of pH on the Formation of 2,3-Butanedione from Riboflavin after Storage under Light. The samples containing riboflavin in 0.1 phosphate buffer with different pH's (4.5, 6.5, and 8.5) were prepared as previously described. The samples were stored under light for 12 h. The quantities of the 2,3-butanedione formed in the samples were analyzed with the standard curves obtained from the added known amount of authentic 2,3-butanedione in each tested buffer solution.

Data Analysis. All of the experiments for the analysis of volatile compounds in the riboflavin solutions were done in triplicate. Data were analyzed using the Microsoft Office Excel program. Comparisons for mean value differences were done by *t*-test. The *p*-value ≤ 0.05 was considered to be significantly different.

RESULTS AND DISCUSSION

Riboflavin Solution Preparation. It was difficult to have a reproducible analysis for the volatile compounds formed from riboflavin solution under light in a preliminary work. The quality of water was extremely important for the determination of volatile compounds from riboflavin solution under light. The stored deionized or distilled water did not give a good reproducible result. The stored water might have absorbed various volatile compounds from environment. The absorbed volatile compounds in the stored water might have interfered with the volatile compounds formed from riboflavin solution under light during gas chromatograph analysis.

Distilled water was first purified by a Millipore purification system with a filter of Progard and then further purified by a Milli-Q Plus ultrapure water system with Purification Pak for the preparation of riboflavin solution. The riboflavin solution prepared with the freshly purified water provided good reproducible gas chromatograms for the volatile compounds of riboflavin solution. It was extremely important to use the freshly purified water for the preparation of riboflavin solution. It is also very important to seal the sample flask with Parafilm for the storage under fluorescence light (3000 Lux). These might explain the difficulties of separation and identification of the volatile compounds formed from riboflavin under light, which have not been reported in the literature.

Analysis of Headspace Volatile Compounds of Riboflavin Solution by SPME-GC. An aqueous solution of riboflavin (0.5 mM) was prepared and stored under light at 3000 Lux for 0, 3, 6, 9, 12, and 15 h. Some of the sample serum bottles were completely wrapped with aluminum foils to protect the riboflavin solution from light. The wrapped serum bottles were designated as the samples stored in the dark. Therefore, there were riboflavin solution serum bottles stored under light and in the dark.

The gas chromatograms of volatile compounds from the riboflavin solutions under light after 0, 3, 6, and 12 h are shown in **Figure 1**. The fresh riboflavin solution without any storage under light or in the dark showed several small gas chromatographic peaks as shown in **Figure 1** at 0 h. The buffer solution without riboflavin also showed the same gas chromatogram of 0 h riboflavin solution. The several small peaks shown in **Figure 1** at 0 h were not from riboflavin. The several peaks at extremely low concentration might be due to the column bleeding or noise signal of the gas chromatograph.

The light produced and increased only one major peak with the retention time of 6.5 min (**Figure 1**). The major peak with the retention time of 6.5 min increased as the storage time under light increased from 0, 3, 6, or 9 to 12 h. The areas of the gas chromatographic peak with the retention time of 6.5 min in the riboflavin solution under light for 0, 3, 6, 9, 12, or 15 h were 0, 2.34 ± 0.57, 6.78 ± 0.33, 10.46 ± 1.01, 12.18 ± 0.80, or 11.71 ± 0.28 (mV × s), respectively. The gas chromatographic peak area at the retention time of 6.5 min from the riboflavin solution under light for 12 h was significantly higher than those for 3, 6, and 9 h at $p < 0.05$. The peak area did not increase as

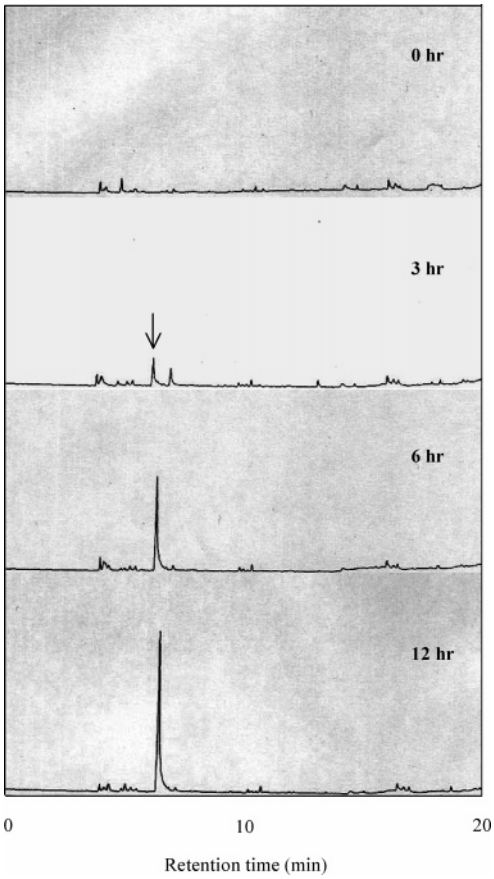

Figure 1. Effects of 0, 3, 6, and 12 h on the volatile compounds from riboflavin solution under light.

the storage time under light increased from 12 to 15 h and no significant difference at $p > 0.05$. The riboflavin solution did not produce any volatile compounds during the storage from 0 to 15 h in the dark (data not shown). The buffer solution without riboflavin under light for 15 h also did not produce any volatile compounds. The results suggested that both riboflavin and light were required to produce the gas chromatographic peak with the retention time of 6.5 min.

Gas Chromatography and Olfactometry. The aqueous riboflavin solution under light had a buttery odor. The odor of the effluent from a sniffing port of gas chromatograph with the retention time of 6.5 min was a buttery flavor. The buttery odor from the gas chromatographic peak at the retention time of 6.5 min was the same odor of the riboflavin solution stored under light. The result suggested that the gas chromatographic peak with the retention time of 6.5 min was responsible for the odor formed from riboflavin solution under light.

Identification of Volatile Compound. The mass spectrum of the gas chromatographic peak with the retention time of 6.5 min is shown in **Figure 2A**. The mass spectrum was tentatively identified as 2,3-butanedione. The gas chromatographic retention time of authentic 2,3-butanedione was 6.5 min. The mass spectrum of the authentic 2,3-butatnedione shown in **Figure 2B** was the same as the mass spectrum of **Figure 2A**. The unknown compound of buttery odor in riboflavin solution with the retention time of 6.5 min was 2,3-butanedione. The authentic 2,3-butanedione was added to the buffer solution at the level of 0.1 μg/mL. The odor characteristics of the buffer solution with the authentic 2,3-butanedione were exactly the same as those of the riboflavin solution under light. The buttery odor compound with the retention time of 6.5 min was positively

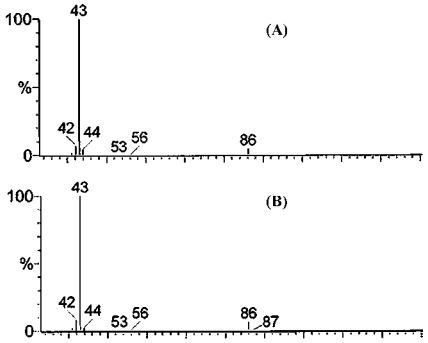

Figure 2. Mass spectra of gas chromatographic peak with the retention time of 6.5 min (**A**) and authentic 2,3-butanedione (**B**).

identified as 2,3-butanedione by the combination of gas chromatographic retention time, mass spectrum, and odor characteristic of authentic 2,3-butadione.

Mechanism for 2,3-Butanedione Formation from Riboflavin under Light. The proposed mechanism for the formation of 2,3-butanedione from the riboflavin under light is shown in **Figure 3**. Riboflavin is a photosensitizer for the formation of singlet oxygen (*4*, *7*, *12*−*14*, *21*). The riboflavin in singlet state absorbs light and becomes the excited singlet state riboflavin. The excited singlet riboflavin becomes the excited triplet riboflavin by intersystem crossing mechanism (*17*). The excited triplet riboflavin reacts with triplet oxygen to form singlet state riboflavin and singlet state oxygen by triplet−triplet annihilation (*17*). Singlet oxygen is an electrophilic molecule and reacts with electron-rich compounds such as riboflavin, linolenic acid, or aromatic amino acids (*20*). Singlet oxygen reacts with riboflavin, which has several double bonds, and then forms riboflavin endoperoxide through 6,9-addition as shown in **Figure 3** (*20*, *22*). The reaction rate between riboflavin and singlet oxygen was very fast at the rate of 1.01×10^{10} M^{-1} s^{-1} (*7*). Singlet oxygen is directly added to the electron-rich double bond of riboflavin and also produces dioxetane through 7,8-cycloaddition (*20*, *22*). The 7,8-dioxetane of riboflavin endoperoxide at 6 and 9 produces 2,3-butanedione by scissions, as shown in **Figure 3**. This mechanism suggested that riboflavin under light was destroyed by singlet oxygen oxidation and produced a volatile compound, 2,3-butanedione.

Effect of Sodium Azide on Volatile Compound Formation of Riboflavin Solution under Light. To study the possibility of singlet oxygen involvement in the formation of the volatile compound from the self-sensitized photooxidation of riboflavin, sodium azide, a well-known singlet oxygen quencher (*23*, *24*), was added to the riboflavin solution. The prepared solution was stored in the light box at 3000 Lux for 12 h. The gas chromatograms of riboflavin solution containing sodium azide are shown in **Figure 4**. As the addition of sodium azide increased from 0, 0.5, and 1.0 to 5.0 mM, the peak sizes of the volatile compound with the retention time of 6.5 min decreased. The gas chromatographic peak areas with retention time of 6.5 min in riboflavin solutions containing 0, 0.25, 0.5, 1.0, or 5.0 mM sodium azide under light for 12 h were 12.37 ± 0.80, 6.30 ± 0.49, 4.35 ± 0.19, 3.19 ± 0.31, or 1.69 ± 0.19 (mV × s), respectively. The addition of 5.0 mM sodium azide reduced 86% of the formation of the volatile compound with the retention time of 6.5 min. This result suggested that singlet oxygen was involved in the formation of the buttery odor compound with the gas chromatographic retention time of 6.5 min in the riboflavin solution under light for 12 h. This also indicated that

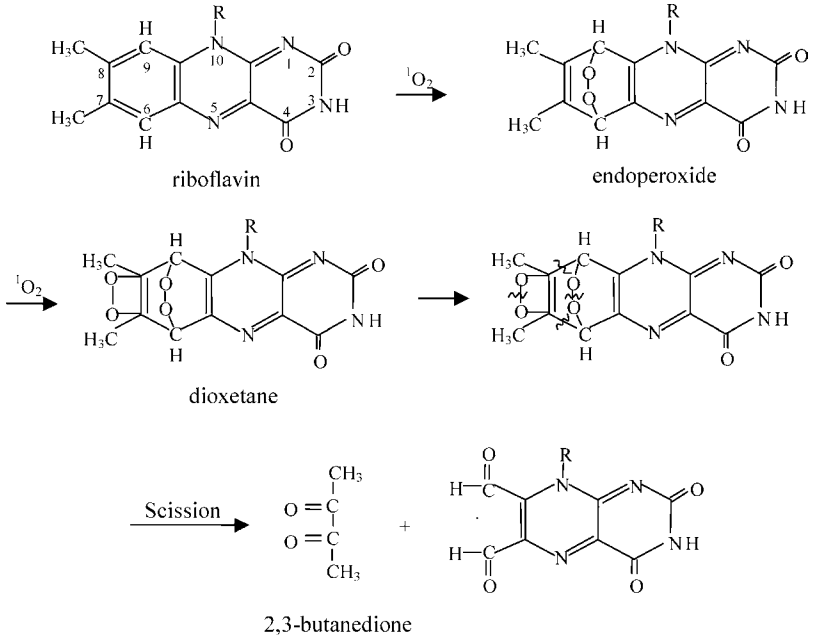

Figure 3. Proposed mechanism for the formation of 2,3-butanedione from riboflavin and singlet oxygen.

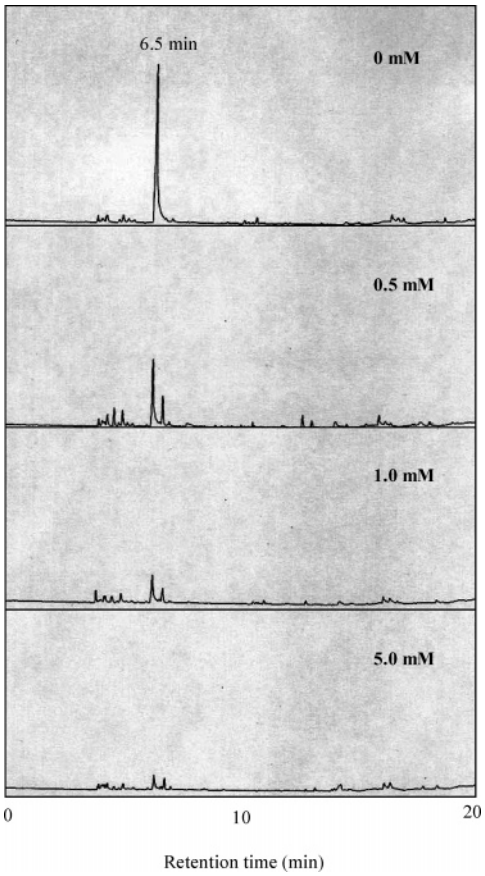

Figure 4. Effects of 0, 0.5, 1.0, and 5.0 mM sodium azide on the volatile compounds from riboflavin solution under light for 12 h.

singlet oxygen reacted with riboflavin to produce 2,3-butanedione, and riboflavin was destroyed by singlet oxygen.

Effect of pH on 2,3-Butanedione Formation from Riboflavin under Light. The quantities of 2,3-butanedione formed from riboflavin in the 0.1 M phosphate buffer with different pH's were analyzed to study the effects of pH on the 2,3-butanedione formation. The quantifications of the 2,3-butanedione in the samples were based on the standard curves obtained

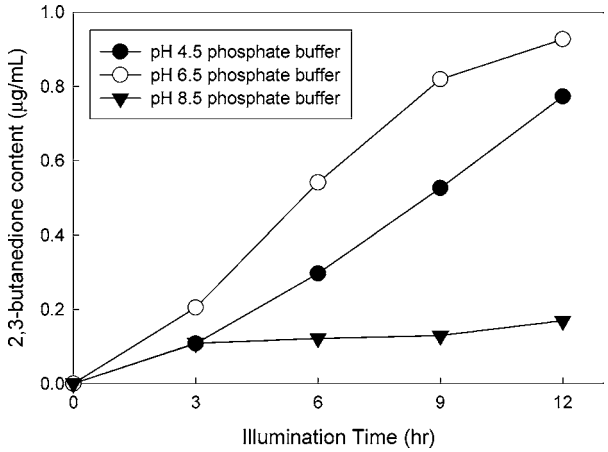

Figure 5. Formation of 2,3-butanedione from riboflavin in 0.1 M sodium phosphate buffer solution with different pH's under light for 12 h.

from the added known amount of authentic 2,3-butanedione in the each tested buffer solution. **Figure 5** shows the quantity of 2,3-butanedione formed from riboflavin in 0.1 M phosphate buffer solution with different pH's (pH 4.5, 6.5, and 8.5) during 12 h light illumination. The pH of the buffer solution greatly affected the formation of 2,3-butanedione from riboflavin. The highest contents of 2,3-butanedione were formed at pH 6.5, followed by at pH 4.5 and 8.5, in a decreasing order. The contents of 2,3-butanedione formed in the buffer solutions with pH 4.5, 6.5, and 8.5 were 0.92 ± 0.005, 0.77 ± 0.053, and 0.17 ± 0.024 μg/mL. It has been previously reported that riboflavin was most stable at pH 6.5, followed by pH 4.5 and 8.5 (*25*). It is interesting to note that the 2,3-butanedione formation was closely related to the stability of riboflavin at the different pH's. The higher is the stability of the riboflavin under light, the higher is the 2,3-butanedione formation from the phosphate buffer. To check the sodium phosphate effect on the 2,3-butanedione formation, the 2,3-butanedione formation from riboflavin in purified water was analyzed and compared to that in 0.1 M phosphate buffer (pH 6.5) after 12 h light illumination. In the purified water, 2,3-butanedione was also the only major volatile compound formed from riboflavin after

light exposure as in the phosphate buffer. Yet, the 2,3-butanedione content (0.82 ± 0.01 μg/mL) formed in the purified water was somewhat lower than that (0.57 ± 0.10 μg/mL) in the phosphate buffer of pH 6.5. The result showed that sodium phosphate did not affect the mechanism for the formation of 2,3-butanedione, but slightly accelerated its formation. We also tested the effects of riboflavin contents in the sodium phosphate buffer (0.1 M, pH 6.5) on the 2,3-butanedione formation. Lower riboflavin concentration in buffered solution induced the significantly lower content of 2,3-butanedione in the solution after 12 h light exposure. The concentrations of 2,3-butanedione formed from 0.25 mM riboflavin and 0.5 mM riboflavin in sodium phosphate buffer solution (pH 6.5) were 0.82 ± 0.01 and 0.53 ± 0.06 μg/mL.

In conclusion, riboflavin in a buffer solution under light produced a buttery odor. The buttery odor compound was positively identified as 2,3-butanedione by a combination of gas chromatographic retention time, mass spectrum, and odor evaluation with authentic 2,3-butanedione. Sodium azide reduced the formation of 2,3-butanedione from riboflavin solution under light. The 2,3-butanedione was formed by the reaction between singlet oxygen and riboflavin. The detailed mechanism for the formation of 2,3-butanedione from riboflavin and singlet oxygen under light was presented. The buttery odor compound from riboflavin under light could affect the flavor quality of foods containing riboflavin under light. This paper reports the formation of buttery odor 2,3-butanedione formed from riboflavin under light for the first time. The previously identified compounds formed from riboflavin under light are nonvolatile lumichrome and lumiflavin (*19*). The formation of nonvolatile lumichrome and lumiflavin and volatile 2,3-butanedione from riboflavin solution under light may explain the rapid destruction of riboflavin in foods under light.

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