Riboflavin-Photosensitized Singlet Oxygen Oxidation Product of Vitamin D_2

Joan M. King[a,*] and David B. Min[b]

[a]Department of Food Science, Louisiana State University Agricultural Center, Baton Rouge, Louisiana 70803, and [b]Department of Food Science and Technology, The Ohio State University, Columbus, Ohio 43210

ABSTRACT: The riboflavin-photosensitized singlet oxygen oxidation of vitamin D_2 in a model system of 12% water and 88% acetone was studied to understand the possible oxidation of fortified vitamin D in milk. Only the samples containing vitamin D_2 and riboflavin under light storage showed two new peaks in the HPLC chromatogram, indicating that singlet oxygen oxidation of vitamin D_2 had occurred. UV analysis indicated that a new compound was formed from the reaction of the triene of vitamin D_2 with oxygen. The mass spectrum showed that one of the two compounds had a molecular ion at $m/z = 412$, which was an increase of the mass of vitamin D_2 by the mass of exactly one oxygen. The IR spectrum suggested the presence of a hydroxyl group and no carbonyl group in the product. The combined information from UV, MS, and FTIR spectra and the chemical mechanisms of singlet oxygen oxidation of vitamin D_2 indicated that a 5,6-epoxide of vitamin D_2 was formed from vitamin D_2 in the presence of riboflavin under light.

Paper no. J10180 in *JAOCS 79,* 983–987 (October 2002).

KEY WORDS: Oxidation product, photosensitization, riboflavin, singlet oxygen, vitamin D_2.

Vitamin D is important for normal mineralization and growth of bones. Both cholecalciferol, D_3, and ergocalciferol, D_2, are used in the fortification of milk and infant formulas to help prevent rickets in children and osteomalacia in adults (1). However, excess vitamin D can cause hypercalcemia, kidney stones, and oxidation of tissue lipids (2). Increased levels of free radicals in animal tissues after dosage with excess levels of vitamin D have been observed by electron spin resonance spectroscopy (3). Intake of antioxidants such as tocopherol are known to reduce the oxidation of vitamin D and the formation of kidney stones (3).

Compounds such as tachysterol, lumisterol, and 7-dehydrocholesterol can be formed through photo-isomerization of vitamin D (4). The conjugated triene structure of vitamin D is susceptible to oxidation. The type of solvent, the wavelength of light used during irradiation, and the presence of a sensitizer (e.g., Rose Bengal) can influence the photochemistry of vitamin D (5–8).

Foods naturally contain sensitizers, such as chlorophyll in vegetable oils (9) and riboflavin in milk (10), that can initiate oxidation by absorbing light to produce singlet oxygen. Singlet oxygen can react directly with electron-rich compounds such as unsaturated FA and with fat-soluble vitamins such as vitamin D, resulting in the degradation of essential nutrients and the formation of possibly toxic compounds (11).

Many studies have been done to test the stability of vitamins in milk. These studies analyzed the photooxidation of vitamins A, C, and riboflavin. Although vitamin A was fairly stable (12), riboflavin was shown to initiate deteriorative reactions in milk, and off-flavors could be formed in milk exposed to light (13). Aurand *et al.* (14) suggested that riboflavin caused the formation of singlet oxygen in milk, since oxidation in milk decreased when a singlet oxygen quencher was added. Berliner *et al.* (10) confirmed that singlet oxygen was formed by riboflavin in milk by using electron spin resonance spectroscopy. Renken and Warthesen (15) found that light did not affect vitamin D loss in an acetonitrile system but did cause loss of vitamin D in skim milk containing riboflavin. The authors hypothesized that the loss of vitamin D was due to photosensitized oxidation by singlet oxygen, but they did not test this hypothesis. King and Min (16) and Li *et al.* (17) showed, in a model system, that singlet oxygen oxidation of vitamin D occurred only in the presence of riboflavin, light, and oxygen and that the singlet oxygen could be quenched by α-tocopherol, ascorbic acid, or carotenoids.

King and Min (16) reported that vitamin D is susceptible to singlet oxygen oxidation and that various metabolites and products of vitamin D may interfere with calcium metabolism or may be toxic. Therefore, the objective of this work was to separate and identify the major product formed from the oxidation of vitamin D_2 by riboflavin-photosensitized singlet oxygen.

EXPERIMENTAL PROCEDURES

Chemical and reagents. Analytical reagent-grade acetone (Mallinckrodt Specialty Chemical Co., Paris, KY) was used for sample preparation. Ergocalciferol (vitamin D_2, >97% purity) and riboflavin were obtained from Sigma Chemical Company (St. Louis, MO). HPLC-grade acetonitrile and methanol were obtained from Fisher Scientific (St. Louis, MO).

Preparation of samples and storage conditions. Samples containing 3,000 ppm of standard vitamin D_2 in a mixture of 12% water and 88% acetone with and without 15 ppm riboflavin were prepared to form a simple mixed-model system in which both riboflavin and vitamin D were soluble.

*To whom correspondence should be addressed at Dept. of Food Science, Louisiana State University Agricultural Center, 111 Food Science Bldg., Louisiana State University, Baton Rouge, LA 70803.
E-mail: jking@agctr.lsu.edu

Prepared samples (16 mL) were transferred into 25-mL serum bottles. The bottles were tightly sealed with Teflon-coated septa and aluminum caps (Supelco, Inc., Bellefonte, PA). Sample bottles were placed in the dark or in a 4,000-lux light storage box at 45°C for 8 or 24 h. Sample concentrations were chosen based on our previous work (17).

Separation of vitamin D_2 oxidation products. HPLC was used to separate the products of vitamin D_2 oxidation. A Hewlett-Packard 1050 high-performance liquid chromatograph (Avondale, PA) with UV detection at 254 nm and an Econosphere C18 column of 3-µm particle size (150 × 4.6 mm; Alltech, Deerfield, IL) was used. Helium was used to sparge the mobile phase prior to starting the pumping system. The mobile phase was a mixture of 50% acetonitrile and 50% methanol at a flow rate of 1 mL/min. The 200 µL of sample was injected with an auto injection system operated with oil-free compressed air.

MS, FTIR, and UV analyses. The samples were repeatedly separated by injecting them multiple times into the chromatograph, and the separated individual peak was collected into a 5-mL brown glass vial. The individual samples collected were evaporated to dryness under a stream of nitrogen. The samples were stored in the dark under nitrogen at 4°C to prevent oxidation until analyzed for identification by spectrometry. MS was performed on a Hewlett-Packard 5971A mass spectrometer. Spectra were obtained by electron ionization at 70 eV and an ion source temperature of 220°C. Samples were dissolved in acetonitrile, and the UV spectrum was obtained by scanning from 190 to 268 nm on a Spectronic 1001 spectrophotometer (Milton Roy, Rochester, NY). The FTIR spectrum was determined with the use of a KBr pellet, made with sample dried from acetone, for transmission analysis using a Mattson Cygnus 100 FTIR (Madison, WI). The resolution was 2 cm^{-1}, and 128 scans were performed for each peak.

RESULTS AND DISCUSSION

Sample analysis by HPLC. The HPLC chromatogram of the fresh sample containing 3,000 ppm vitamin D_2 only (without riboflavin) is shown in Figure 1A. This indicated that the vitamin D_2 was not a pure compound. Photo-isomerization of vitamin D_2 can occur during storage (5). Because the vitamin D_2 obtained from Sigma was formed by irradiation of ergosterol, the B, D, and F peaks of Figure 1A may be photo-isomers of vitamin D_2. The HPLC chromatogram of sample containing 3,000 ppm vitamin D_2 with 15 ppm riboflavin held for 8 h in darkness was essentially the same as that of Figure 1A and so is not shown here. The HPLC chromatogram of sample containing 3,000 ppm vitamin D_2 in the absence of 15 ppm riboflavin stored for 8 h in light was also essentially the same as that of Figure 1A and is not shown. Both vitamin D_2 with 15 ppm riboflavin in darkness and vitamin D_2 without riboflavin under light were stable for 8 h. The presence of 15 ppm riboflavin and storage time had no effect on vitamin D_2 stability during storage in darkness.

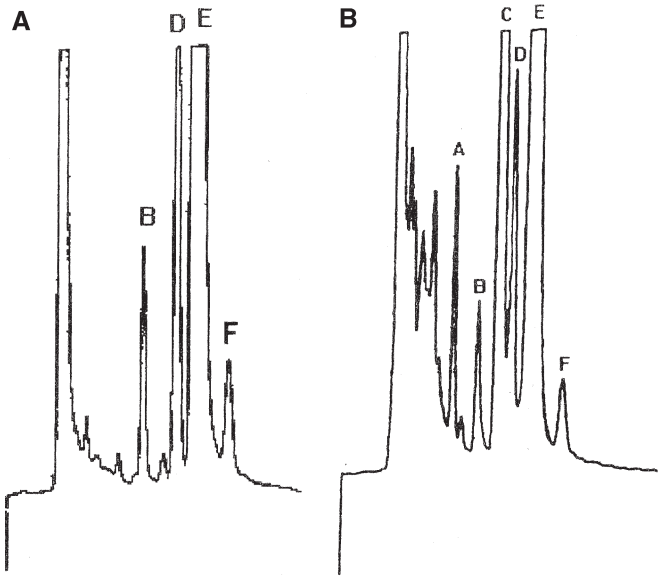


FIG. 1. (A) HPLC chromatogram of 3,000 ppm fresh vitamin D_2 without riboflavin in 12% water and 88% acetone; (B) HPLC chromatogram of 3,000 ppm vitamin D_2 and 15 ppm riboflavin in 12% water and 88% acetone under light storage for 8 h. All peak retention times are noted in Table 1.

Two new peaks, A and C, were present in the HPLC chromatogram of the sample containing 3,000 ppm vitamin D_2 and 15 ppm riboflavin that was exposed to light for 8 h (Fig. 1B). Our previous research (16) showed that vitamin D_2 was oxidized by riboflavin-photosensitized singlet oxygen in the sample containing riboflavin and stored in the light box. Support for riboflavin-induced singlet oxygen formation in light and in the presence of air was reported by Berliner *et al*. (10).

For peak E of Figure 1B, which was later positively identified as vitamin D_2 by MS and which had the HPLC retention time of the authentic compound, the vitamin D_2 content decreased by 33 and 52% as the storage time increased from 0 to 8 and 24 h in light storage (Table 1). However vitamin D_2 did not decrease as the storage time increased from 0 to 8 h in darkness. The peak C area in Figure 1B increased 160-fold from 0 to 8 h and then an additional 15.6% as the storage time increased from 8 to 24 h in light. These results indicate that photosensitized oxidation of vitamin D_2 occurred only in the presence of riboflavin, light, and oxygen in our study. The

TABLE 1
Average HPLC Percentage Peak Area (based on total chromatogram area) of Duplicate Vitamin D_2 Samples Stored in the Light for 8 and 24 h

Retention time (min)[a]	Vitamin D_2 alone (% control)	15 ppm riboflavin	
		Light 8 h (%)	Light 24 h (%)
6.27 (A)	0.04	0.61	1.52
7.72 (B)	0.53	0.34	0.22
8.97 (C)	0.03	4.79	5.54
9.70 (D)	1.16	0.80	0.84
10.9 (E)	29.8	20.0	14.2
12.9 (F)	0.24	0.16	0.13

[a]Letters correspond to the peaks in Figure 1.

presence of an extremely small area for peak C retention time in the pure vitamin D_2 sample could be attributed to a photosensitized singlet oxygen oxidation product of vitamin D_2 formed during the production of vitamin D_2 from ergosterol by irradiation during manufacturing. The singlet oxygen was most likely formed from residual plant chlorophyll in vitamin D_2, as chlorophyll is a strong sensitizer of singlet oxygen.

Analyses of peaks C and E by MS, UV, and FTIR. The maximum absorbances of peak C (Fig. 1B) and vitamin D_2 were 250 and 264 nm, respectively. The absorbance at 264 nm was due to the conjugated triene of vitamin D_2. The maximum absorbance of acyclic conjugated diene was between 215 and 230 nm (18). For peak C the maximum absorbance of 250 nm indicated that this peak did not have a conjugated triene structure but that it still had at least two double bonds. These two double bonds were not conjugated double bonds, which would have a maximum absorbance at around 215 to 230 nm. Therefore, the singlet oxygen must have reacted with one of the double bonds at carbons 5 and 6 or 7 and 8. If the singlet oxygen had reacted with double bonds at carbons 7 and 8, the new compound would have had a conjugated diene with maximum absorbance between 215 and 230 nm. The UV spectrum showed that it did not have conjugated double bond absorbance at this range of wavelengths. The UV spectrum strongly suggested that the singlet oxygen reacted with the double bond of the conjugated triene of vitamin D_2 at carbons 5 and 6.

The FTIR spectrum of peak C is shown in Figure 2. The strong absorption band at around 3400 cm^{-1} was due to the functional group of an alcohol OH stretching vibration. The shape and relative strength of absorbance of the OH group to the other functional group absorbances of the vitamin D_2 spectrum indicated that peak C most likely had one alcohol group in the molecule. The absorbance at 2900 cm^{-1} was due to C–H stretching in vitamin D_2. The medium-size absorbance at 1711 cm^{-1} was due to nonconjugated carbon–carbon double bond stretching of vitamin D_2 (18). It was not due to the absorbance of a carbonyl group because the absorbance in this range was not strong enough for a carbonyl group compared to absorbance of the OH group at around 3400 cm^{-1}. It would be very difficult to form a ketone or aldehyde from the double bonds of carbon 5 and 6 or of 7 and 8 with singlet oxygen. Therefore, the possibility of the presence of a carbonyl group at peak C was eliminated. Epoxide ring bonds showed stretching at 1250 cm^{-1}, and the C–C bond of the ring showed stretching at 810 to 950 cm^{-1}, whereas a third band was observed at the 750 to 840 cm^{-1} range (18). We observed weak to medium bands at each of these regions. The IR spectrum indicated that peak C of Figure 1B had only one OH group and double bonds, but a carbonyl group was not present according to comparisons of absorbances with vitamin D_2.

The molecular ion, M^+, of peak E was at m/z = 396, which is the actual mass of vitamin D_2. The fragment at m/z = 271 represents the loss of the side chain of mass = 125, whereas m/z = 253 includes the loss of the side chain plus the loss of water (Figs. 3A and B). The fragment at m/z = 136 represents the mass of the A ring up to position 7 with a molecular formula of $C_9H_{12}O$, and the fragment at m/z = 118 includes the loss of water and $C_9H_{12}O$ (Figs. 3A and B). The retention

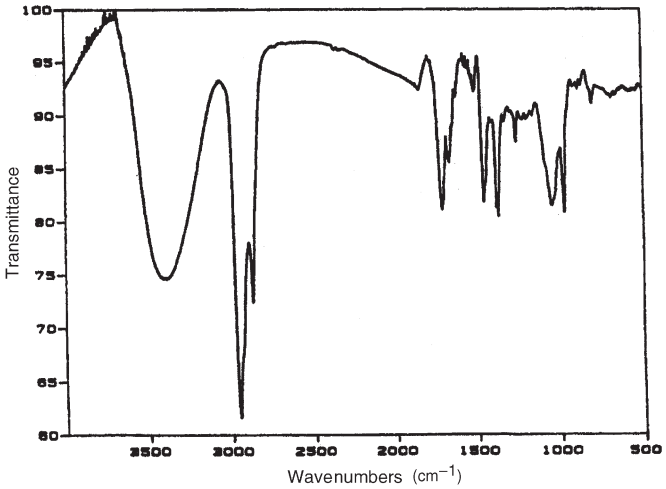


FIG. 2. FTIR spectrum of peak C of Figure 1B.

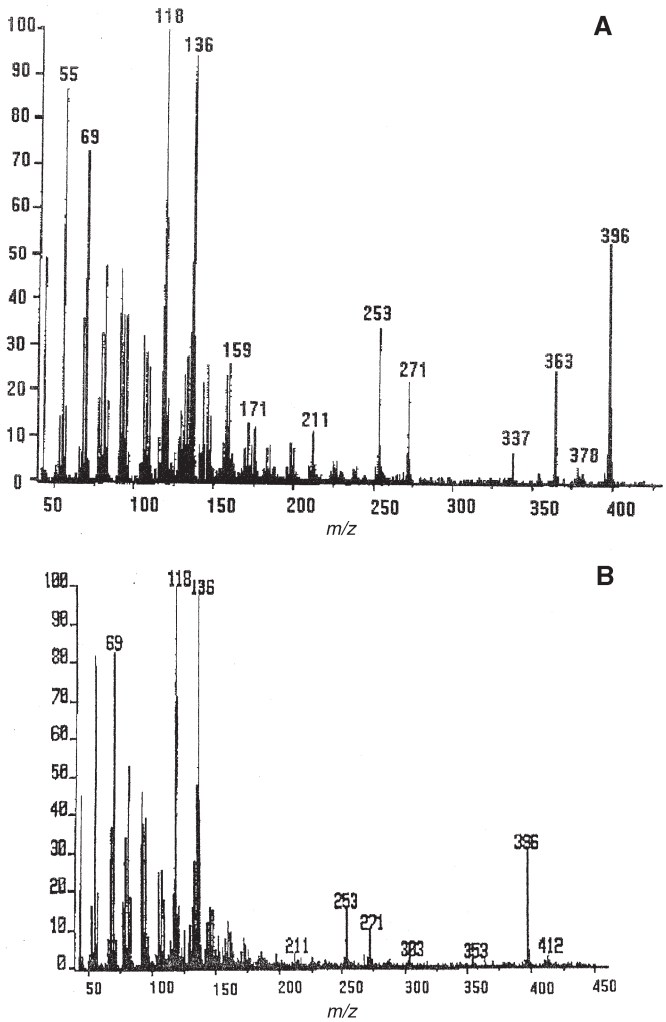
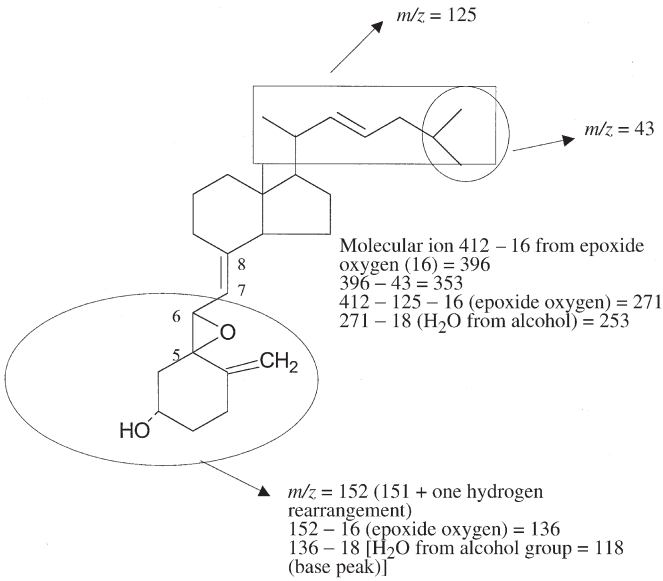


FIG. 3. (A) Mass spectrum of HPLC peak E of Figure 1B; (B) mass spectrum of peak C of Figure 1B.

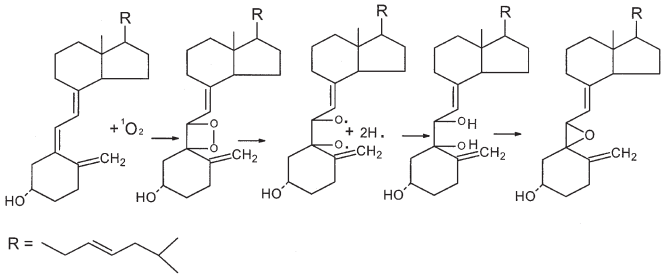


SCHEME 1

time of peak E was the same as that of the authentic vitamin D_2. The mass spectrum of peak E was essentially the same as the MS analysis of vitamin D_2 reported by Elliott and Waller (19). Based on the UV, IR, and MS data in this study as compared to the literature, peak E was identified as vitamin D_2.

The mass spectrum of peak C is shown in Figure 3B. The spectrum also showed fragments at *m/z* = 396, 271, 253, 136, and 118. However, mass fragments at *m/z* = 396, 271, and 253 for peak C had a lower intensity than those of peak E. Both peaks C and E had a base peak at *m/z* = 118. The molecular ion of peak C was *m/z* = 412, which is an increase of the mass of vitamin D_2 (396 g/mol) by the mass of exactly one oxygen (16 g/atom). The molecular ion at *m/z* 412 strongly suggested that peak C was a product oxidized by adding one oxygen atom to vitamin D_2. Matsueda and Katsukura (20) observed a loss of mass of 16 for an $(M^+ - O)$ fragment in the mass spectral analysis of an epoxide of cholesta-5,7-dien-3β-ol. The mass spectrum of peak C suggested that the compound was an epoxide of vitamin D_2. The singlet oxygen, which was formed by riboflavin in the sample, could easily react with the double bond of vitamin D_2 to form an epoxide, as shown in Scheme 1. The electrophilic singlet oxygen would react directly with the electron-rich double bonds of compounds (21).

The logical mechanism for the formation of epoxide from vitamin D_2 and singlet oxygen during oxidation is shown in Scheme 2. The information from MS, UV, and IR spectra and the mechanism for the chemical reaction of vitamin D_2 with singlet oxygen showed that peak C is a 5,6-epoxide of vitamin D_2 formed from the reaction of singlet oxygen with vitamin D_2. This research also showed that 33 and 53% of vitamin D_2 was lost during 8 and 24 h of storage (Table 1). Based on this study and those of others, milk should not be exposed to light during processing and storage because the vitamin D_2 present may be susceptible to oxidation by riboflavin-photosensitized singlet oxygen.



SCHEME 2

ACKNOWLEDGMENTS

Salaries and research support were provided by state and federal funds appropriated to the Ohio Agricultural Research and Development Center, The Ohio State University.

REFERENCES

1. Parish, D.B., Determination of Vitamin D in Foods: A Review, *CRC Crit. Rev. Food Sci. Nutr. 12*:29–57 (1979).
2. Jacobus, C.H., M.F. Holick, Q. Shao, T.C. Chen, I.A. Holm, J.M. Kolodny, G.E.H. Fuleihan, and E.W. Seely, Hypervitaminosis D Associated with Drinking Milk, *N. Eng. J. Med. 326*: 1173–1177 (1992).
3. Spirichev, V.B., and I.N. Sergeev, Vitamin D: Experimental Research and Its Practical Application, *World Rev. Nutr. Diet. 56*: 173–216 (1988).
4. Jacobs, H.J.C., and E. Havinga, Photochemistry of Vitamin D and Its Isomers and of Simple Trienes, in *Advances in Photochemistry*, edited by J.N. Pitts, G.S. Hammond, and K. Gollnick, John Wiley & Sons, New York, 1979, pp. 305–373.
5. Gielen, J.W.J., R.B. Koolstra, H.J.C. Jacobs, and E. Havinga, Triplet-Sensitized Interconversion and Photooxygenation of Vitamin D and *trans*-Vitamin D, *Recl. J. R. Neth. Chem. Soc. 99*: 306–311 (1980).
6. Jacobs, H.J.C., J.W.J. Geilen, and E. Havinga, Effects of Wavelength and Conformation on the Photochemistry of Vitamin D and Related Conjugated Trienes, *Tetrahedron Lett. 22*: 4013–4016 (1981).
7. Yamada, S., K. Nakayama, and H. Takayama, Synthesis of 6,19-Epidioxy-9,10-secoergosta-5(10),7,22-trien-3-ols from Vitamin D Derivatives by Oxidation with Singlet Oxygen, *Ibid. 49*: 4895–4898 (1978).
8. Moriuchi, S., F. Tsuruki, Y. Otawara, N. Hosoya, S. Yamada, K. Nakayama, and H. Takayama, Biological Activity of the Endoperoxides Derived from Vitamin D Derivatives by Dye-Sensitized Photooxidation, *J. Nutr. Sci. Vitaminol. 25*:455–458 (1979).
9. Lee, S.H., and D.B. Min, Effects, Quenching Mechanism and Kinetics of Carotenoids in Chlorophyll-Sensitized Photooxidation of Soybean Oil, *J. Agric. Food Chem. 38*:1630–1634 (1990).
10. Berliner, L.J., D.G. Bradley, D. Meinholtz, D.B. Min, and T. Ogata, The Detection of Riboflavin Photosensitized Singlet Oxygen Formation in Milk by Electron Spin Resonance, in *Supramolecular Structure and Function*, edited by G. Pifat, Balaban Publishers, Rehovot, Israel, 1994, pp. 67–77.
11. Neff, W.E., E.N. Frankel, E. Selke, and D. Weisleder, Photosensitized Oxidation of Methyl Linoleate: Secondary and Volatile Thermal Decomposition Products, *Lipids 17*:780–790 (1982).
12. Thompson, J.N., and P. Erdody, Destruction by Light of Vitamin A Added to Milk, *Can. Inst. Food Sci. Tech. J. 7*:157–161 (1974).

13. Aurand, L.W., J.A. Singleton, and B.W. Noble, Photooxidation Reactions in Milk, *J. Dairy Sci. 49*:138–143 (1966).
14. Aurand, L.W., N.H. Boone, and G.G. Giddings, Superoxide and Singlet Oxygen in Milk Lipid Peroxidation, *J. Dairy Sci. 60*: 363–369 (1977).
15. Renken, S.A., and J.J. Warthesen, Vitamin D Stability in Milk, *J. Food Sci. 58*:552–556, 566 (1993).
16. King, J.M., and D.B. Min, Riboflavin Photosensitized Singlet Oxygen Oxidation of Vitamin D, *Ibid. 63*:31–34 (1998).
17. Li, T.L., J.M. King, and D.B. Min, Quenching Mechanisms and Kinetics of Carotenoids in Riboflavin Photosensitized Singlet Oxygen Oxidation of Vitamin D_2, *J. Food Biochem. 24*:477–492 (2000).
18. Dyer, J.R., *Applications of Absorption Spectroscopy of Organic Compounds,* Prentice Hall, Englewood Cliffs, NJ, 1985.
19. Elliott, W.H., and G.R. Waller, Vitamins and Cofactors, in *Biochemical Applications of Mass Spectrometry*, edited by G.H. Waller, Wiley Interscience, New York, 1972, pp. 507–510.
20. Matsueda, S., and Y. Katsukura, Antitumour-Active Photochemical Oxidation Products of Provitamin D, *Chem. Ind. 12*: 411 (1985).
21. Bradley, D.G., and D.B. Min, Singlet Oxygen Oxidation of Foods, *CRC Crit. Rev. Food Sci. Nutr. 31*:211–236 (1992).

[Received December 10, 2001; accepted June 11, 2002]