On-Line Photostability Evaluation System for Chemiluminescence Detection of Phloxine (R-104)

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A simple and rapid on-line analytical system was configured for the evaluation of the photostability of various food coloring agents constantly exposed to light. The screening test for photostability of food coloring agents was carried out in the system consisting of 60 cm of Teflon tubing for ultraviolet (UV) irradiation with a flow rate of mobile phase (water/acetonitrile = 1:1, v/v) at 1 mL/min. When the products derived from photochemical reaction of phloxine (R-104), which is recognized to exhibit chemiluminescence induced by UV irradiation, were separated, several peaks were confirmed on the chromatogram in this system. Furthermore, the peak of chemilumigenics observed on the chromatogram of photon-counting chemiluminescence detection corresponds to the new peaks on the UV chromatogram. Also, the presence of phenoxy radical ($g = 2.0061$) formed by UV irradiation of R-104 was confirmed with an electron spin resonance spectrometer, indicating a reasonable relationship ($r = 0.9041$) between the formation of free radical and the chemiluminescence phenomenon.

Keywords: *Photostability; phloxine; chemiluminescence; on-line system; ESR; free radical formation*

INTRODUCTION

Although some chemicals are guaranteed of their safety through various toxicological examinations by biological assay, the safety of their secondary products formed by physicochemical factors, such as light and heat, has not been evaluated sufficiently until now. Therefore, physicochemical stabilities of chemicals in pharmaceuticals and foods that are directly consumed in the body need to be elucidated in the aspect of safety.

Meanwhile, interesting findings have confirmed that various pharmaceuticals, as represented by imipramine, exhibit chemiluminescence (CL) induced by irradiation (Milofsky and Birks, 1990). The CL coupled with flow injection analysis (FIA) and high-performance liquid chromatography (HPLC) has made progress as a detection method of high sensitivity along with development of a CL detection system (Niederlander et al., 1991). Especially, the CL-HPLC system has improved its sensitivity practically as much as 200 times compared with the ultraviolet (UV) detection method. In fact, there have been several studies utilizing this CL phenomenon for evaluation method of stabilities of pharmaceuticals (Sato et al., 1986, 1988; Imaizumi et al., 1989). However, there are no reports of a simple method for the evaluation of the photostability of food colors. The food color R-104 was recognized to exhibit ≈10-fold intensity of irradiated CL of imipramine hydrochloride (Mizugaki et al., 1985; Ishimitsu et al., 1995). Thus, the formation of CL and free radicals induced by UV irradiation (Suzuki et al., 1992) and its safety ought to be investigated.

In this paper, an exploratory investigation of the photochemical reaction mechanism of irradiated CL of food colors is presented along with configuration of the system for evaluation of photostability.

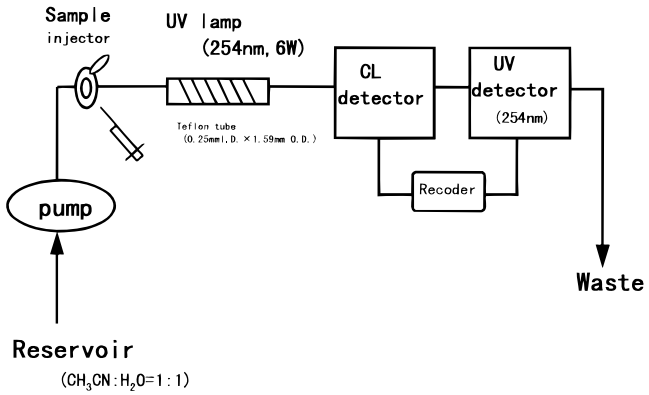

Figure 1. Schematic diagram of the flow system to detect photostability of food colors.

MATERIALS AND METHODS

Reagents. Food coloring agents, erythrosine (R-3), phloxine (R-104), rose bengal (R-105), acid red (R-106), brilliant blue FCF (B-1), indigo carmine (B-2), tartrazine (Y-4), and sunset yellow FCF (Y-5), were obtained from Daiwa Kasei (Yono, Japan). Eosin, fluorescein sodium salt, eosin bluish, 2′,7′-dichlorofluorescein sodium salt, and gallein were purchased from Tokyo Chemical Industry, Co. Ltd. (Tokyo, Japan), and imipramine hydrochloride was obtained from Sigma Chemical Co. (St. Louis, MO). Disodium hydrogen orthophosphate (Na_2HPO_4), sodium dihydrogen orthophosphate (NaH_2PO_4), and acetonitrile used in mobile phase were all of analytical grade purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Water was obtained using a Milli-Q system (Nippon Millipore Ltd., Tokyo, Japan).

Apparatus. A schematic diagram of the flow system for the evaluation of the photostability is shown in Figure 1. The flow system consists of a Shimadzu (Kyoto, Japan) LC-9A pump for the delivery system of mobile phase, a Soma (Hinode-machi, Japan) S-3400 CL detector, a Shimadzu (Kyoto, Japan)

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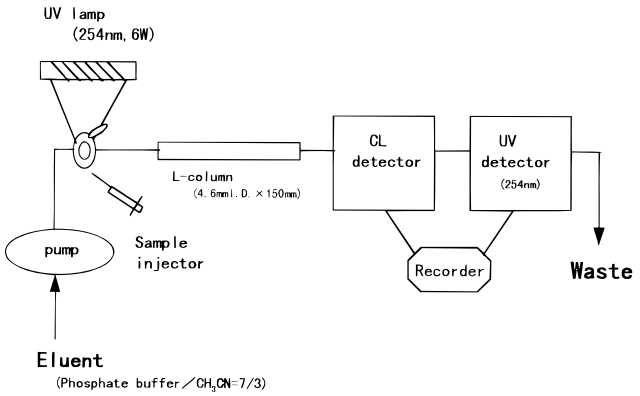

Figure 2. Schematic diagram of on-line analytical system to evaluate the light sensitivity of food colors.

SPD-6A UV−vis detector, and a Toshiba (Tokyo, Japan) GL-6 (6 W) sterilizing lamp with coiled Teflon tubing (0.25 mm i.d., 1.59 mm o.d.). The UV intensity was measured by Topcon UVR-254. Also, as shown in Figure 2, the modified flow system consists of a mounted irradiation port on a sample loop injector (Rheodyne Model 7125) so as to set the irradiation time accordingly and a HPLC column (L-column, 4.6 mm i.d. × 150 mm, Association of Chemical Inspection, Tokyo, Japan). In this system a photon-counting CL detector (CLD-110, Tohoku Electronics Industries Co., Ltd., Sendai, Japan) was used to replace the conventional CL detector to provide better sensitivity. Further, a photodiode array detector (SPD-M6A, Shimadzu, Kyoto, Japan) was used for measuring the UV−vis spectra of reaction products.

Measurement of Irradiated Reaction Product. The separation of the irradiated reaction products has been achieved by ODS column with the UV−vis detector at 254 nm and a photon-counting CL detector. The column used was an L-column with an acetonitrile/50 mM phosphate buffer (pH 7.0) (3:7, v/v) as a mobile phase, at 1.0 mL/min of the flow rate.

Free Radical Measurement. The free radicals were measured with electron spin resonance (ESR) (JES-RE1X, JEOL, Tokyo, Japan) after irradiation of R-104. Two hundred microliters of 100 μM R-104 was taken into test tube and irradiated with a UV lamp (13.9 μW/cm^2) for 30 s, and the ESR spectrum was measured in a ESR flat cell. ESR conditions were as follows; center field, 336.5 mT; power, 20 mW; modulation frequency, 100 kHz; response time, 0.03 s; sweep width, 5 mT; temperature, ambient.

RESULTS AND DISCUSSION

Screening Test of Food Colors and Similar Colors. Using the on-line system mentioned above, the capability of this system was evaluated by carrying out a screening test of various colors. Figure 3 shows the chemical structures that have chemiluminescence after UV irradiation, and Table 1 summarizes their chemiluminescence intensities. R-104 exhibited the strongest CL of all the colors tested, and its intensity is nearly 10 times that of imipramine. Several colors of xanthene skeleton and with halogen substituent also exhibited relatively strong CL intensity. Reasonably strong CL intensity exhibited by colors with a chloride group suggests the electronegative substituent plays a major role in the CL phenomenon in food colors.

Preliminary Examination. The behavior of R-104 exhibiting a strong CL by irradiation was monitored. The various concentrations of R-104 were irradiated with UV (254 nm, 290 μW/cm^2) at 6 W in 5.3 cm^2 area, and the change in CL derived from R-104 was determined by photon counter. After irradiation, the CL of R-104 was degraded rapidly and disappeared after 30 min. The CL was not observed for concentrations >1 mM R-104.

Table 1. Comparison of Chemiluminescence Intensity after UV Irradiation[a]

food color	relative Cl intensity: nonirradiated	relative Cl intensity: irradiated
imipramine hydrochloride	ND	0.60
erythrosine (R-3)	ND	0.28
phloxine (R-104)	ND	9.70
rose bengal (R-105)	ND	3.28
acid red (R-106)	ND	0.15
eosin	ND	0.25
fluoresceine salt[b]	1.60	4.20
eosin bluish	0.11	0.71
2′,7′-dichlorofluorescein salt[b]	ND	3.89
gallein	ND	0.15
brilliant blue FCF (B-1)	ND	ND
indigo carmine (B-2)	ND	ND
tartrazine (Y-4)	ND	ND
sunset yellow (Y-5)	ND	ND

[a] Flow rate, 1 mL/min; concentration, 1 mM; irradiation intensity, 718.3 μW/cm^2; sample volume, 5 μL. ND, no detection. [b] Sodium salt.

Optimization of On-Line Photostability Evaluation System. Considering the degradation of CL, optimum operational conditions for the on-line photostability evaluation system based on the FIA method were investigated as in following sections to achieve maximum intensity of CL with high precision. Acetonitrile enhances the CL intensity of the sample, and water/acetonitrile (1:1, v/v) was applied for screening the polar substances.

Effect of Irradiation Time. The length of Teflon tubing using irradiation section was varied in the range of 30−180 cm (corresponds to 2−12 s at a flow rate of 1 mL/min). Figure 4 shows the relationship between the length of Teflon tubing and the CL intensity and/or UV intensity. While UV intensity remained the same, the CL intensity increased with increasing irradiation time. However, increase in irradiation time also caused scattering and poor reproducibility of CL intensity. Therefore, to obtain maximum reproducibility, 60 cm of Teflon tubing was chosen and applied for all subsequent studies.

Effect of Concentration. The changes in CL intensity according to various sample concentrations are shown in Figure 5. Although the UV intensity linearly increased with an increase in sample concentration, the maximal CL intensity was almost attained with the concentration of 1 mM.

Elucidation of Photochemical Reaction of R-104. *Detection of Irradiated Reaction Products of R-104.* R-104 was analyzed using the on-line photostability evaluation system. One of the Tar group coloring agents, R-104 effectively colors materials at small quantities and is widely used in foods and beverages because it is more stable and economical than natural color. However, there are no regulations on Allowable Daily Intake (ADI), and safety concerns over this food color have grown with reports of teratogenic effects on pregnant ICR mouse (Seno et al., 1984) and mutagenicity being activated photochemically (Yoshikawa et al., 1978). Therefore, knowledge of the physicochemical stability against UV irradiation including free radical formation of R-104 is also valuable from a food hygiene chemistry point of view.

UV chromatograms in Figure 6 show the difference before and after the irradiation reaction of R-104. Several new peaks, conceivably of photoreaction prod-

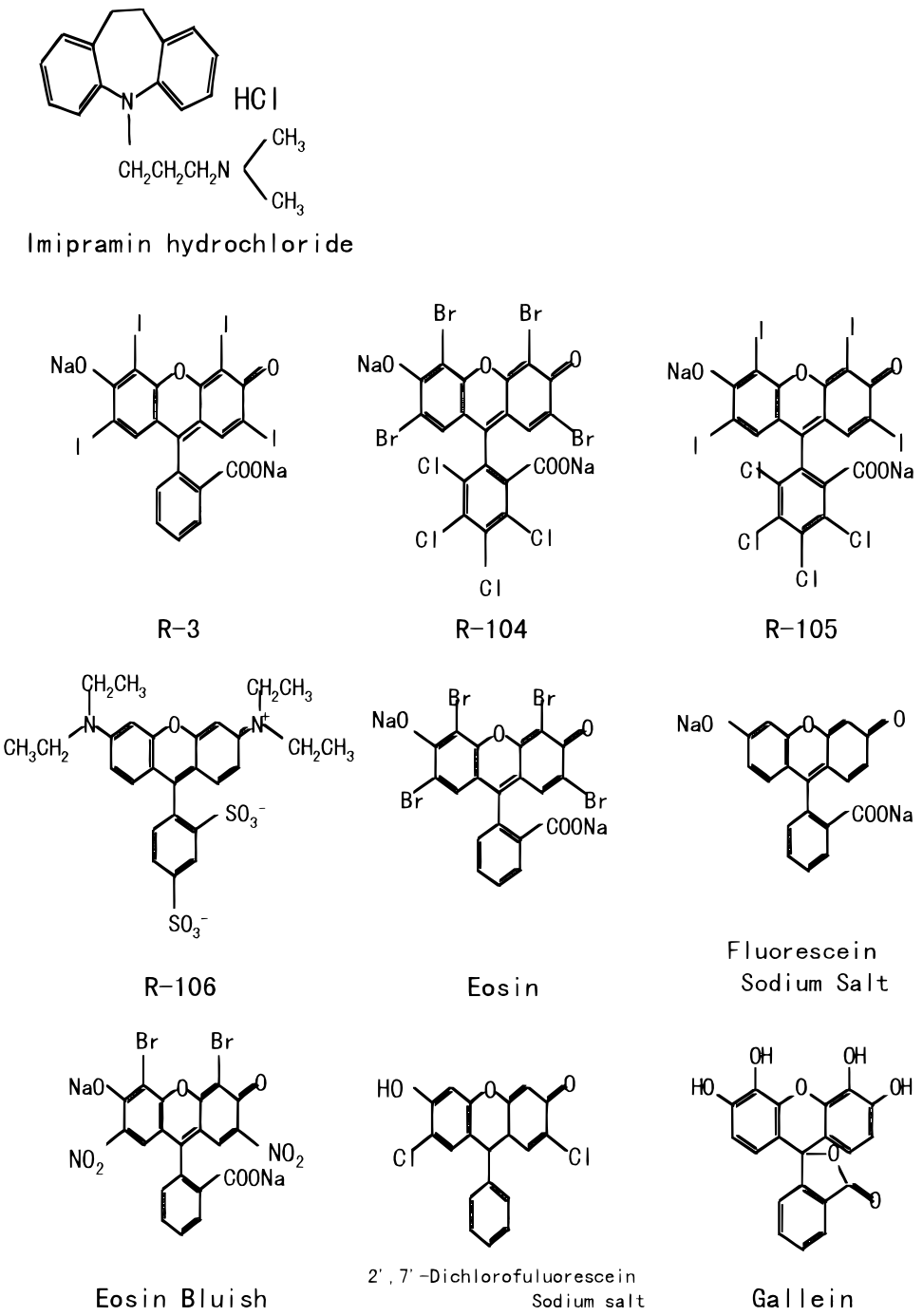

Figure 3. Structures of investigated chemicals with chemiluminescence by UV irradiation.

ucts, were confirmed on the UV chromatogram after irradiation. Peak H, with a retention time of ~10 min, corresponds to primary color agent R-104 and decreased after irradiation, upon which several new peaks were observed having retention times of 2−3 and 5−6 min. Photoreaction products presumably possess higher polarity than R-104 itself according to their retention times.

Presence of Reaction Intermediates Derived from Photochemical Reaction. The CL chromatogram with photon-counting CL detector was examined, noting newly observed peaks on the UV chromatogram. As shown in Figure 7, peaks A and B observed on the UV chromatogram after irradiation were detected on the CL chromatogram (peaks A* and B*), which implies that the peaks of high polarity appearing on the UV chromatogram participate in CL. Figure 8 shows the comparison of intensities of peaks A and B (peaks A* and B*) obtained from both UV and CL chromatograms over the irradiation time. Both peaks A and B indicated rapid change of intensities within 1 min. As for peak B, the intensity increased first but decreased steadily with irradiation time on both chromatograms of UV and CL, suggesting that peak B observed on the UV chromatogram participates in CL. On the other hand, it became clear that while the UV intensity of peak A increased with irradiation time on the UV chromatogram, the CL intensity of peak A* decreased on the CL chromatogram. Although peaks A and A* detected on the UV and CL chromatograms, respectively, can be considered to have similar chromatographic behaviors because of their retention times, peak A increased while peak A* decreased with time, which suggests the

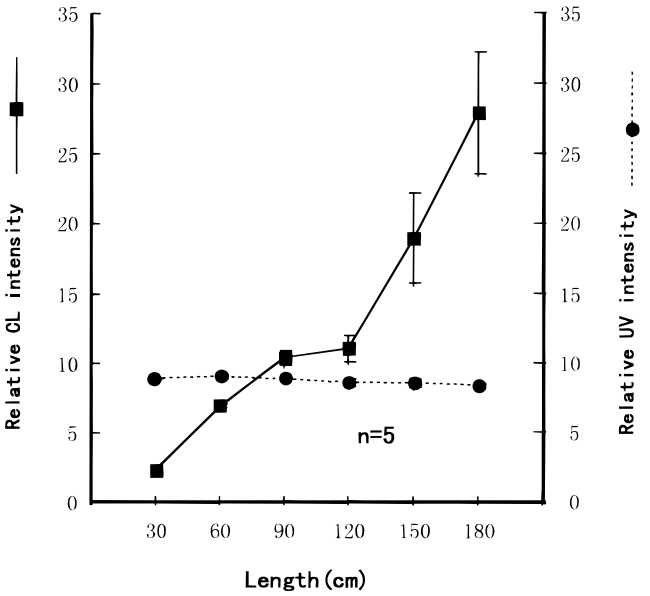

Figure 4. Change in sensitivity by varying the length of Teflon tubing: sample, R-104; concentration, 1 mM; flow rate, 1 mL/min; irradiation intensity, 718.3 μW/cm^2.

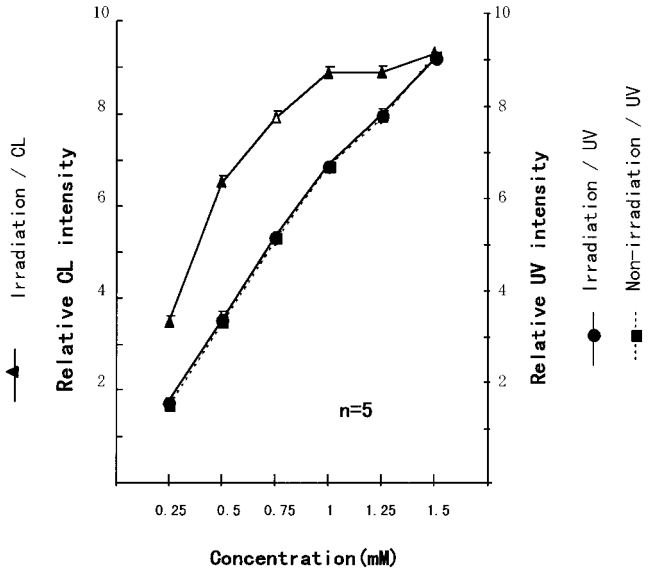

Figure 5. Effect of concentration of R-104 on sensitivity: flow rate, 1 mL/min; irradiation time, 2 s; irradiation intensity, 718 μW/cm^2.

possibility that peak A* on the CL chromatogram corresponds to a reaction intermediate exhibiting CL.

Analysis with Photodiode Array Detector and Emission Spectrum. The UV detector equipped within the On-line photostability evaluation system was replaced by a photodiode array detector with which R-104 was measured at a wavelength range between 200 and 600 nm, and the changes in three-dimensional spectrochromatograms (wavelength/time/absorption) of reaction products after irradiation were investigated. The chromatographic spectra of both primary color agent R-104 and peak B, considered to be a photoreaction product, showed identical maximum absorptions on each UV and visible region (240 nm, 537 nm), showing no significant spectrum differences; thus, it can be concluded that there are no basic structural differences between them. In contrast, the spectrum of peak A showed absorption in the UV region but not in the visible region, which indicates the loss of the basic skeleton, and therefore the color agent is thought to have gone through significant structural changes. Furthermore, absorption of peaks D and E was recognized in both UV and visible regions, indicating the possibility of the presence of accompanying color agents.

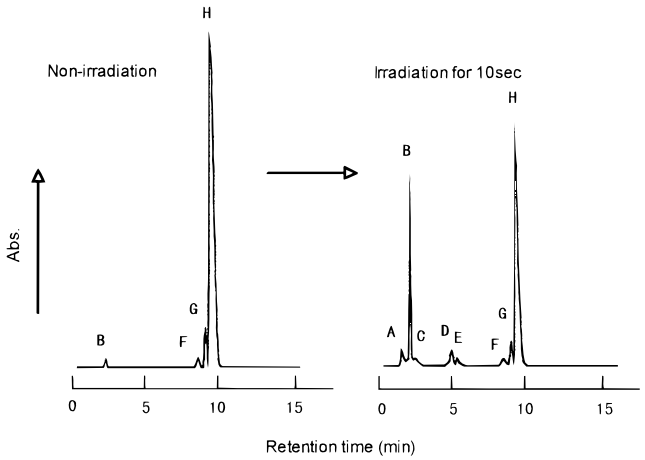

Figure 6. Effect of UV irradiation of R-104 on UV chromatograms.

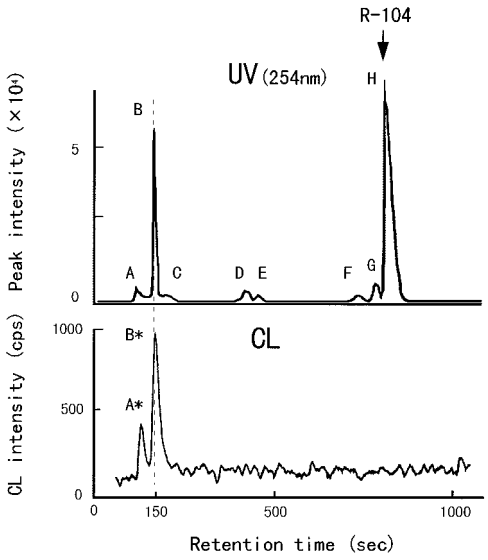

Figure 7. HPLC chromatogram of R-104 after UV irradiation for 10 s.

Irradiation-Induced CL and Free Radicals. Having observed irradiated CL on substances in previous experiments, free radical formation induced by UV irradiation has been detected in aqueous solutions (Rutgi and Reisz, 1978) and on the skin surface (Ushujima et al., 1985) with the ESR spectrometer. There have been several interesting papers on the correlation between free radicals and CL such as CL formation during transition process of $^1O_2$ to ground state oxygen (Takahashi et al., 1990) and CL of free radicals formed by UV irradiation of imipramine. On the basis of the ESR spectrum ($g = 2.0061$) and the chemical structure of R-104, we speculate the formation of phenoxy radicals with UV irradiation. This reaction was presumably due to either hydrogen radicals acting as electron donors in aqueous solution or hydroxyl radicals. Also, this ESR signal continues to increase with UV irradiation time and is relatively stable. Correlation between phenoxy radical and CL derived from irradiated R-104 was examined, and good correlation ($r = 0.9041$) was obtained between them; therefore, CL can be considered to participate in free radical formation. Generally, one of the properties of ESR measurement is the capability

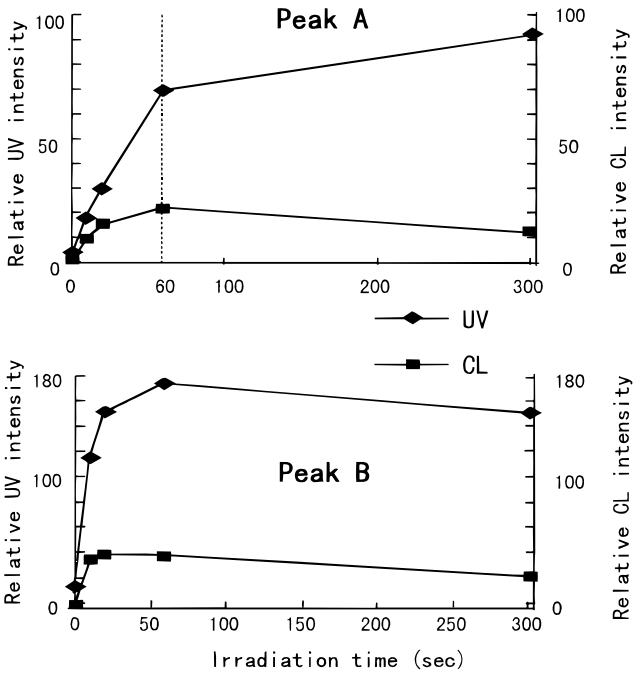

Figure 8. Effect of irradiation time on photodegradation of R-104.

of detecting free radicals directly, but it is not easily applicable for this screening test because it lacks not only sensitivity and quantitation ability but also accessibility due to the costly instrument. Nonetheless, it is expected to be a valuable technique to detect free radicals during the process of radical formation exhibiting CL.

Conclusions. R-104 used in this study conceivably decomposed into the substances of peaks A−G on the UV chromatogram after the chemical reactions induced by UV irradiation. Also, the substance corresponding to peak B is considered to be a phenoxy radical, demonstrating an absorption spectrum identical with that of R-104 and indicating a stable free radical under ESR detection. Again, simple and rapid evaluation of chemical photostability was possible to perform with good precision using this newly configured system.

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