Estimation of Irradiation Doses of Raw Beef Liver Samples using 5,6-Dihydrothymidine as an Irradiation Marker

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ABSTRACT: The quantities of (5S)- and (5R)-5,6-dihydrothymidine (total DHdThd) in frozen beef liver samples were compared between those irradiated by an electron beam (EB) and those subjected to γ-irradiation. DNA extracted from the samples was enzymatically digested to nucleosides and analyzed by liquid chromatography–tandem mass spectrometry for total DHdThd and 2’-deoxythymidine (dThd). Total DHdThd was formed radiospecifically and dose-dependently and the dose–response curves of the ratio of total DHdThd to dThd (total DHdThd/dThd) were similar for both the EB- and γ-irradiated samples. The total DHdThd/dThd was stable after long-term storage (4 months) at −20 °C and the followed heat treatment in a microwave oven. The total DHdThd/dThd could be a robust marker and is equally effective at quantitating both EB- and γ-ray irradiation history. The irradiation doses of raw beef liver samples were estimated using the dose–response curves of the total DHdThd/dThd of other irradiated samples. The ratio of the estimated dose to the actual dose was 0.74–1.30 in the irradiation range of 4.67–7.62 kGy.

INTRODUCTION

Food irradiation may improve hygiene compliance and shelf life by controlling microorganisms. Electron beam (EB), γ -ray, and X-ray are used as irradiation sources.1–3 Food irradiation is approved in >50 countries, and the quantity of irradiated foods was 405 000 tons in 2005.4,5 In the U.S., irradiation up to 7.0 kGy is approved for pathogen control in meat products without heat treatment.6 In Japan, however, irradiation is approved only to delay sprouting in potato. Nevertheless, irradiation has recently been considered for the elimination of pathogens in raw beef liver products. Raw beef liver dishes (liver sashimi) and yukhoe (raw beef dishes) have been popular items at barbecue restaurants in Japan. Nevertheless, the food-borne illness caused by enterohemorrhagic Escherichia coli (EHEC) has occurred sporadically as a result of consuming these raw beef dishes. Pursuant to a severe EHEC outbreak resulting from the ingestion of contaminated raw beef meat or liver dishes in 2011,7 the Ministry of Health, Labour, and Welfare (MHLW) of Japan revised the edible raw meat standards in the same year to improve the trimming process.8 In 2012, MHLW banned the sale of raw beef liver products for liver sashimi and revised the Food Sanitation Act because the reduction of the risk of EHEC contamination in the beef liver was effectively impossible. The bacterial pathogens may reside in the biliary ducts as well as the surface of the livers.8 MHLW also stated that it was prepared to lift the ban on the sale of raw beef liver products provided that technologies are developed to confirm that EHEC contamination has been eliminated. Irradiation is a promising candidate, and investigations into its efficacy at eradicating EHEC from raw beef liver products are ongoing. Irradiation history detection methods are required to control food irradiation properly. Therefore, various methods have been developed.9 Ten official standard methods for the determination of food irradiation history have been established by Le Comité Européen de Normalisation (CEN). For fatty meat samples, method no. EN1785, which detects the radiolytic fat products 2-alkylcyclobutanones (ACBs), is appropriate.10 However, this method must be modified for use with low-fat liver and lean meats. For example, the test solutions may have to be concentrated.11 DNA is universally contained in the low-fat liver and lean meats, and its content would not be affected by the difference of individual animals. Therefore, we have developed and presented a new method for determining beef liver irradiation histories by detecting of the sum of (5S)- and (5R)-5,6-dihydrothymidine (total DHdThd) as irradiation-induced modified nucleosides in DNA.12 Total DHdThd is a radiolytic product of 2’-deoxythymidine (dThd)13 and was a target antigen for the enzyme-linked immunosorbent assay of shrimp irradiation history.14 In this method, the ratio of total DHdThd to thymidine (total DHdThd/dThd) in DNA is measured by liquid chromatography coupled to tandem mass spectroscopy (LC–MS/MS) as a marker for irradiation history. We also improved the speed and efficiency of this method.15 It can

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successfully determine the irradiation histories of ground beef and shrimp. The total DHdThd/dThd dose—response curves nearly overlapped, even though the samples were derived from different individual animals. Furthermore, the total DHdThd/dThd of irradiated samples was stable even after freezing storage for ≥100 days. This exhibit that total DHdThd/dThd would not be affected by the differences of quality of DNA obtained from samples.

In certain irradiation history determination methods, the markers (signals and peak responses) are affected by the differences in sample composition and irradiation source. For the thermoluminescence (TL)\textsuperscript{16} and photostimulated luminescence (PSL)\textsuperscript{17} methods, the TL glow ratio and the PSL signal counts vary with the sample silicate content even when the same kinds of foods are being analyzed.\textsuperscript{6,19} The analysis output also varied with the irradiation source.\textsuperscript{5,20} Gadgil et al. also reported that the relative efficiency of ACB formation in raw beef patties would differ between EB- and γ-irradiated samples.\textsuperscript{21} However, variations in the marker type between methods should not hinder irradiation history determination and may provide information about the sample production areas. Nevertheless, these variations make it difficult to estimate the actual irradiation dose of the sample when it is compared with a standard sample whose irradiation dose has been certified. EN1786 is an electron spin resonance (ESR) method used to detect the free radicals formed in meat with bone in response to irradiation.\textsuperscript{22} EN1786 may estimate the irradiation dose by extrapolation, which involves stepwise-dosed sample reirradiation.\textsuperscript{23} Using the currently available technology, it would be difficult to determine post-shipment food irradiation doses. However, total DHdThd/dThd is a robust irradiation history marker that may be able to estimate sample irradiation doses by comparing them with standard samples. To examine the ability of this method, we compared total DHdThd/dThd between EB- and γ-irradiated raw beef liver samples, examined the effects of heat treatment before DNA extraction on total DHdThd/dThd, and then estimated the irradiation doses of raw and heat-treated beef liver samples using the dose—response of other raw beef liver samples.

**MATERIALS AND METHODS**

**Beef Liver Samples.** Fresh beef liver (BL) samples derived from three animals were obtained from three retail markets in Osaka, Japan on different days and were designated as Liver I, Liver II, and Liver III, respectively. The organs were sliced into plates and wrapped in a food-grade poly(vinylidene chloride) (PVDC) film. The length, width, height, and weight of each plate were ~40 mm, 20 mm, 5 mm, and 10 g, respectively. The plates were placed in a plastic dish (100 mm diameter, 20 mm height; AGC Techno Glass Co. Ltd., Shizuoka, Japan) and stored at −20 °C until irradiation.

**Chemicals.** Total DHdThd was purchased from Berry & Associates Inc. (Dexter, MI). The standard for total DHdThd consisted of 85% (SS)- and 15% (SR)-isomers and the total DHdThd was expressed as the sum of these isomers. Sodium iodide (NaI), dThd, proteinase K, and nuclease P1 were obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Alkaline phosphatase (ALP) and snake venom phosphodiesterase 1 (SVPD) were obtained from Roche Diagnostics GmbH (Mannheim, Germany) and Sigma-Aldrich Corp. (St. Louis, MO), respectively. The other chemicals used were the highest commercially available grade.

**Irradiation.** γ-Irradiation was conducted at the radioisotope centers of Osaka Prefecture University, Japan, using 60Co (2.7 kGy/h). EB-irradiation was conducted at the Kumatori Works of Nuclear Fuel Industries, Ltd. (Tokyo, Japan). The planned irradiation doses were 0.5, 1, 2, 5, and 8 kGy, respectively. The EB energy was 10 MeV, and the irradiation rates at each planned dose were 4 M Gy/h (0.5 kGy), 8 M Gy/h (1 kGy), 16 M Gy/h (2 kGy), and 40 M Gy/h (5 and 8 kGy), respectively. The wrapped livers were placed in the plastic dish described above and stored at −20 °C at least overnight until immediately before irradiation. Both γ- and EB irradiation were conducted in a freezing mixture consisting of ice and NaCl (−20 to −17 °C) and on dry ice, respectively. The actual irradiation doses were determined with radiochromic film dosimetry patches (FWT-60-1P; Far West Technology, Inc., Goleta, CA) placed on both sides of the dishes (total two radiochromic films placed per a dish). To avoid the influence of humidity, each film was placed in a plastic bag. The batch calibration of the film was performed at −20 °C before using the films by irradiating them with already calibrated alanine dosimeters (Harwell Dosimeters, Oxfordshire, U.K.) from 0 kGy up to 11 kGy.

After irradiation, the samples were stored at −20 °C until further analysis. DNA was extracted within 22 days after irradiation, except for part of Liver III. The parts of Liver III, which were EB- or γ-irradiated, were stored at −20 °C for >4 months before DNA extraction (Liver III stored) or heat-treated prior to DNA extraction (Liver III heated) to determine the stability of total DHdThd/dThd to storage and heat treatment. The analysis of total DHdThd in each BL sample, including Liver III stored and Liver III heated, was conducted three times.

**Heat Treatment of Beef Liver Samples.** The frozen samples were thawed in a refrigerator (4 ± 1 °C) overnight and ∼10 g samples were heat-treated in a 150 mL glass beaker covered with a PVDC film in a conventional microwave oven (NE-N2; Panasonic Corp., Osaka, Japan) at 170 W for 180 s. The core temperatures of the samples were measured with a food thermometer. Changes in the sample weight following heat treatment were recorded to estimate moisture loss.

**DNA Extraction.** Sample DNA was extracted according to the method described by Wang et al., with minor modifications.\textsuperscript{25} Briefly, 1.0 g samples of the raw or heat-treated beef livers were placed in a 15 mL polypropylene (PP) tube (BD Biosciences, San Jose, CA) containing 2 mL lysis solution consisting of 10 mM Tris–HCl (pH 7.5), 5 mM MgCl\textsubscript{2}, 0.32 M sucrose, and 1% Triton X-100. The samples were homogenized with a TissueRuptor (QIAGEN, Venlo, The Netherlands) and centrifuged at 10 000 g and 4 °C for 1 min. The supernatant was removed and the sediment was rinsed twice with lysis solution and resuspended in 2 mL extracting solution consisting of 10 mM Tris–HCl (pH 8.0), 5 mM ethylenediaminetetraacetic acid, and 1% sodium dodecyl sulfate. Then, 1 μL RNase A solution (20 µg/mL) was added and the samples were incubated at 37 °C for 10 min. Next, 80 μL proteinase K solution (20 mg/mL) was added to the samples and they were incubated at 55 °C for 60 min. For the heat-treated samples, the suspensions were also shaken and centrifuged at 10 000 g and 4 °C for 10 min with 2 mL chloroform to remove impurities.

After centrifugation of nonheat-treated samples or heat-treated samples, the supernatant was transferred to a new 15 mL PP tube, to which 3 mL NaI was added. The tube was then
Table 1. Storage Periods of the Beef Livers Prior to DNA Extraction and Their Yields

| storage period (days) | irradiation source | BL samples | DNA yield (average ± SD; mg/g) |
|-----------------------|-------------------|------------|-------------------------------|
|                       | EB                | I          | II                           |
|                       |                   | III        | III stored                   | III heated |
|                       |                   |            | 128                          | 141        |
|                       |                   | 65         | 180                          |
|                       | γ                 | 65         | 250                          |
|                       |                   | 127        | 175                          |
|                       |                   | 1.8 ± 0.4  | 1.8 ± 0.4                    |
|                       |                   | 3.4 ± 0.3  | 3.4 ± 0.3                    |
|                       |                   | 3.1 ± 0.1  | 3.1 ± 0.1                    |

*EB, electron beam; BL, beef liver. Average and standard deviations (SDs) calculated from nonirradiated and irradiated samples (n = 6).*

**RESULTS**

**DNA Extraction from Beef Liver Samples.** The post-irradiation BL sample storage periods and the sample DNA yields are listed in Table 1. The DNA extraction time and the average yield were ~5 h and ~2.4–3.4 mg/g, respectively. The DNA yields for the EB- and the γ-irradiated BL samples were very similar. Obvious change in DNA yield was not observed even after >4 months storage at ~20 °C (Liver III stored).

However, heat treatment reduced DNA yield in the Liver III (Liver III heated) by nearly 50% relative to the liver sample before heat treatment. After heat treatment, the Liver III heated sample darkened in color and its weight was ~65% that of the sample before heat treatment because of moisture loss. Chloroform had to be added to the heat-treated liver samples to remove the dark-colored impurities. The time required to extract the DNA from the heat-treated samples and their average DNA yield were ~6 h and ~1.8 mg/g, respectively.

**Total DhDThd/dThd in the EB- and γ-Irradiated Beef Liver Samples.** Total DhDThd was formed in a dose- and radiation-dependent manner both for the EB and γ-irradiated BL samples. For nonirradiated BL samples, detectable peaks (signal to noise ratio >3) of (5S)- and (5R)-5,6-dihydrothymidine were not observed. Chromatograms of 4.87 kGy EB and 4.73 kGy γ-irradiated Liver I are shown in Figure 1 and they do not visibly differ between samples. The ratio of the (5S)-isomer to (5R)-isomer was ~85:15 in both samples. The dose-response curves of the total DhDThd/dThd as a function of irradiation dose were similar for EB- and γ-irradiated Liver I, Liver II, and Liver III (Figure 2). Therefore, the total DhDThd/dThd did not vary with the irradiation source. The total DhDThd/dThd dose-response curves for EB- and γ-irradiated samples were also similar for Liver I, Liver II, and Liver III.

**Stability of Total DhDThd/dThd in the Irradiated Beef Liver Samples.** The total DhDThd/dThd dose-response curves for the EB- and γ-irradiated BL samples were nearly constant after a 4 months freezing storage and heat treatment (Figure 3). The core temperature of the samples heat-treated by a microwave oven (Liver III heated) was >75 °C at 45 s and 90 °C at 60 s. After 180 s of microwave heating, the temperature at the center of the sample was steady at 90–95 °C. The total DhDThd generated from the DNA of heat-treated samples was only ~55% that of the untreated samples (Liver III). The dThd produced from the DNA of the sample was also only ~55% that of the untreated samples. Therefore, total DhDThd/dThd was virtually unchanged.

**Estimation of the Irradiation Dose of Beef Liver Samples.** The dose-response curves were similar for the irradiated BL samples that were heat-treated and for those that were frozen for long-term storage. Therefore, the estimation of the irradiation dose of samples from the dose–response curve of other samples was examined. The dose–response curve of EB-irradiated Liver I was adopted as a calibration curve. The parameters (a, b, and r²) of this calibration curve of eq 1 were 0.297, 0.025, and 0.997, respectively. The ratios of the estimated irradiation doses calculated by eq 1 to the actual irradiation doses are listed in Table 2. Estimation error decreased with the increasing irradiation dose. The ratio in the low irradiation range (0.25–1.93 kGy) and the high irradiation range (4.67–7.62 kGy) were 0.23–1.77 and 0.74–
For analysis, test solutions were used undiluted for LC–MS/MS analysis. Retention times of (5′-deoxythymidine (dThd); m/z 245 → 117) and (E−H) 2′-deoxythymidine (dThd; m/z 243 → 127) Peaks S and R in (A) correspond to (5′)-DHdThd and (5R)-DHdThd, respectively. For total DHdThd analysis, test solutions were used undiluted for LC–MS/MS analysis. Retention times of (5′)-DHdThd, (5R)-DHdThd, and dThd were 4.8, 5.5, and 7.4 min, respectively. For total DHdThd analysis, test solutions were used undiluted for LC–MS/MS analysis. For dThd analysis, test solutions were first diluted 1.0 × 10^3-fold with water before LC–MS/MS. Standard solutions of (A) total DHdThd (20 ng/mL) and (E) dThd (50 ng/mL); (B, F) nonirradiated Liver I; (C, G) electron beam (EB)-irradiated Liver I at 4.87 kGy; and (D, H) γ-irradiated Liver I at 4.73 kGy.

Figure 1. Multiple reaction chromatograms monitoring (A–D) (5S)- and (5R)-5,6-dihydrothymidine (total DHdThd; m/z 245 → 117) and (E–H) 2′-deoxythymidine (dThd; m/z 243 → 127). Peaks S and R in (A) correspond to (5S)-DHdThd and (5R)-DHdThd, respectively. Retention times of (5S)-DHdThd, (5R)-DHdThd, and dThd were 4.8, 5.5, and 7.4 min, respectively. For total DHdThd analysis, test solutions were used undiluted for LC–MS/MS analysis. For dThd analysis, test solutions were first diluted 1.0 × 10^3-fold with water before LC–MS/MS. Standard solutions of (A) total DHdThd (20 ng/mL) and (E) dThd (50 ng/mL); (B, F) nonirradiated Liver I; (C, G) electron beam (EB)-irradiated Liver I at 4.87 kGy; and (D, H) γ-irradiated Liver I at 4.73 kGy.

Figure 2. Dose–response curves of the total DHdThd/dThd in irradiated Liver I, Liver II, and Liver III. Open symbols, ○ (red box), and (blue tilted square open) represent EB-irradiated Liver I, Liver II, and Liver III, respectively. Closed symbols, ● (red box solid), and (blue tilted square solid) represent γ-irradiated Liver I, Liver II, and Liver III, respectively. All symbols represent averages of three independent experiments after DNA digestion and LC–MS/MS analysis. Each bar represents standard deviation (n = 3). Inset shows the magnified area of graph indicating the low irradiation dose (0–1.0 kGy). Total DHdThd: the sum of (5S)-DHdThd and (5R)-DHdThd/dThd: 2′-deoxythymidine; EB: electron beam.

Figure 3. Dose–response curves of total DHdThd/dThd in Liver III stored frozen for >4 months (Liver III stored) and heat-treated Liver III (Liver III heated). Open symbols, △ and (green triangle down open) represent EB-irradiated Liver III stored and Liver III heated, respectively. Closed symbols, (blue triangle up open) and (red triangle down open) represent γ-irradiated Liver III stored and Liver III heated, respectively. Each bar represents standard deviation (n = 3). The broken line corresponds to correlation curves plotted by least square methods from EB-irradiated Liver III processed 6 days after irradiation. Functions (γ: total DHdThd/dThd; α: irradiation dose, kGy) and coefficients of correlations (r) of the fitting lines were as follows: y = 0.325x + 0.055 (r², 0.959). Inset shows the magnified area of graph for low irradiation dose (0–2.0 kGy). Total DHdThd: the sum of (5S)-DHdThd and (5R)-DHdThd/dThd: 2′-deoxythymidine; EB: electron beam.

1.30, respectively. The average and SEM of the 10 ratios calculated at each planed irradiation dose (0.5, 1, 2, 5, and 8 kGy) were 0.78–1.11 and 0.03–0.15, respectively.

**DISCUSSION**

The efficacy of this method depends on the amount and quality of DNA extracted from the samples. In a previous report, phenol–chloroform extraction and 50% ethanol precipitation were adopted to extract DNA from BL samples. This procedure extracts fair quantities of DNA from BL samples (~1 mg/g). However, the entire process required ≥2 days. To reduce DNA extraction time and labor, we used NaI as a chaotropic agent to extract DNA in the present study. In this way, the DNA yield was ~2–3x higher than that of the former method and the extraction time was reduced to 5–6 h. Debris generated from sample heat treatment was removed by chloroform wash. This methodology may be the most suitable for DNA extraction from BL samples to determine irradiation histories.

Total DHdThd may be a radiospecific product from dThd as it is not detected in nonirradiated BL samples and is also not formed during experimental processes including DNA extraction and its digestion to nucleosides to prepare the test solution for LC–MS/MS analysis. One of the well-studied modified nucleosides formed by irradiation of DNA, 8-oxo-2′-deoxyguanosine (8-oxo-dGuo), can be formed via oxidation with reactive oxygen species during the experimental processes including DNA extraction and its digestion to nucleosides to prepare the test solution for LC–MS/MS analysis. One of the well-studied modified nucleosides formed by irradiation of DNA, 8-oxo-2′-deoxyguanosine (8-oxo-dGuo), can be formed via oxidation with reactive oxygen species during the experimental process.
Table 2. Ratio of Estimated to Actual Irradiation Dose

| BL samples | irradiation source | period (process) before DNA extraction | average irradiation dose (kGy) | irradiation dose (kGy) |
|------------|-------------------|---------------------------------------|------------------------------|------------------------|
|            |                   |                                       | calculated\(^d\)             | 0.5  | 1    | 2    | 5    | 8    |
| I          | EB                | 22 days (none)                        | 0.29 (0.02)                  | 0.74 (0.10)            | 1.82 (0.02) | 4.45 (0.17) | 7.71 (0.65) |
|            |                   |                                       | 0.25 (0.01)                  | 0.71 (0.02)            | 1.70 (0.01) | 4.87 (0.09) | 7.46 (0.80) |
|            | ratio\(^e\)       |                                       | 1.16 (0.01)                  | 1.03 (0.01)            | 1.07 (0.02) | 0.91 (0.01) | 1.03 (0.00) |
| I          | γ                 | 22 days (none)                        | 0.14 (0.01)                  | 0.61 (0.02)            | 1.13 (0.09) | 3.49 (0.11) | 6.24 (0.80) |
|            |                   |                                       | 0.46 (0.01)                  | 0.83 (0.04)            | 1.31 (0.12) | 4.73 (0.01) | 7.62 (0.25) |
|            | ratio\(^e\)       |                                       | 0.30 (0.01)                  | 0.73 (0.04)            | 0.87 (0.02) | 0.74 (0.01) | 0.82 (0.00) |
| II         | EB                | 13 days (none)                        | 0.37 (0.01)                  | 0.88 (0.04)            | 2.62 (0.12) | 4.68 (0.01) | 9.34 (0.25) |
|            |                   |                                       | 0.39 (0.01)                  | 0.69 (0.04)            | 1.48 (0.12) | 5.08 (0.01) | 7.20 (0.25) |
|            | ratio\(^e\)       |                                       | 0.97 (0.02)                  | 1.27 (0.03)            | 1.77 (0.02) | 0.92 (0.01) | 1.30 (0.00) |
| II         | γ                 | 13 days (none)                        | 0.15 (0.02)                  | 0.72 (0.03)            | 1.16 (0.07) | 4.43 (0.21) | 6.76 (0.44) |
|            |                   |                                       | 0.46 (0.02)                  | 0.83 (0.03)            | 1.31 (0.07) | 4.73 (0.21) | 7.62 (0.44) |
|            | ratio\(^e\)       |                                       | 0.32 (0.01)                  | 0.87 (0.03)            | 0.90 (0.07) | 0.94 (0.01) | 0.89 (0.00) |
| III        | EB                | 6 days (none)                         | 0.45 (0.03)                  | 0.92 (0.03)            | 2.13 (0.09) | 4.32 (0.13) | 8.96 (0.22) |
|            |                   |                                       | 0.29 (0.01)                  | 0.79 (0.03)            | 1.39 (0.09) | 4.92 (0.09) | 7.53 (0.21) |
|            | ratio\(^e\)       |                                       | 1.56 (0.01)                  | 1.16 (0.05)            | 1.53 (0.09) | 0.88 (0.03) | 1.19 (0.00) |
| III        | γ                 | 6 days (none)                         | 0.37 (0.01)                  | 0.74 (0.05)            | 1.96 (0.09) | 3.83 (0.30) | 6.83 (0.21) |
|            |                   |                                       | 0.29 (0.01)                  | 0.79 (0.03)            | 1.39 (0.09) | 4.92 (0.03) | 7.53 (0.21) |
| III        | γ                 | 128 days (none)                       | 0.31 (0.07)                  | 0.70 (0.01)            | 1.80 (0.20) | 3.81 (0.25) | 7.95 (0.49) |
|            |                   |                                       | 0.29 (0.01)                  | 0.79 (0.01)            | 1.39 (0.02) | 4.92 (0.01) | 7.53 (0.20) |
| III        | γ                 | 141 days (heated)                     | 0.31 (0.07)                  | 0.70 (0.01)            | 1.80 (0.20) | 3.81 (0.25) | 7.95 (0.49) |
|            |                   |                                       | 1.08 (0.01)                  | 0.88 (0.03)            | 1.30 (0.15) | 0.77 (0.04) | 1.06 (0.00) |
| III        | γ                 | 127 days (none)                       | 0.11 (0.01)                  | 0.80 (0.03)            | 1.38 (0.15) | 4.83 (0.49) | 8.19 (0.20) |
|            |                   |                                       | 0.48 (0.02)                  | 0.75 (0.08)            | 1.93 (0.11) | 4.67 (0.30) | 7.56 (0.38) |
| III        | γ                 | 175 days (heated)                     | 0.24 (0.02)                  | 0.71 (0.04)            | 1.57 (0.08) | 4.26 (0.28) | 8.14 (0.14) |
|            |                   |                                       | 0.48 (0.02)                  | 0.75 (0.04)            | 1.93 (0.12) | 4.67 (0.28) | 7.56 (0.14) |
|            | ratio\(^e\)       |                                       | 0.36 (0.02)                  | 0.92 (0.04)            | 0.76 (0.02) | 0.95 (0.01) | 0.98 (0.00) |
|            |                   |                                       | 0.48 (0.02)                  | 0.75 (0.04)            | 1.93 (0.12) | 4.67 (0.28) | 7.56 (0.14) |
|            | ratio\(^e\)       |                                       | 0.50 (0.02)                  | 0.95 (0.05)            | 0.81 (0.01) | 0.91 (0.02) | 1.08 (0.00) |
|            | average (SEM) of the 10 ratios\(^h\) | | 0.78 (0.15)                  | 0.98 (0.05)               | 1.11 (0.01) | 0.88 (0.03) | 1.03 (0.05) |

\(^a\)EB, electron beam; BL, beef liver; SD, standard deviation; SEM, standard error of mean. \(^b\)EB, irradiated by electron beam; γ, irradiated by γ-ray. \(^c\)Storage period at ~20 °C from irradiation until DNA extraction; process: heating with microwave at 170 W for 180 s prior to DNA extraction. \(^d\)Determined by calculation from irradiation rates of irradiation sources. \(^e\)Average (SD) irradiation doses calculated from the equation obtained from the dose–response curve of EB-irradiated Liver I (n = 3), γ = 0.297x + 0.025; γ: total DHdThd/dThd; x: irradiation dose (kGy). \(^f\)Average irradiation dose calculated from radiocromic film dosimetry (n = 2). \(^g\)Ratio of averages of calculated to actual irradiation doses. \(^h\)Average and SEM of the 10 ratios (EB or γ irradiated BL samples; Liver I, Liver II, and Liver III including Liver III stored and Liver III heated).

The ratio of (SS)-DHdThd to (SR)-DHdThd was ~85:15 in both the EB and γ irradiated BL samples for all irradiation doses. The reaction mechanism for the conversion of dThd in aqueous solution to total DHdThd by irradiation is reportedly as follows: 26–31 DHdThd reacts with the dThd anion radical, following protonation at the C6 position to form the S6-dihydrothymidine-5-yl radical (S-yl radical), and the addition of hydrogen at C5 of the 5-yl radical to give (SS)-DHdThd or (SR)-DHdThd. The production efficiency of either (SS)- or (SR)-DHdThd depends on further radical reactions occurring at the 5-yl radical. The production efficiency of (SS)-DHdThd may be advantageous compared to that of (SR)-DHdThd in the DNA double helix. In the case of irradiation of dThd solution, the production efficiency of either (SS)- or (SR)-DHdThd was almost the same. 22 In this study, the isomer ratio was almost the same between the γ- and the EB-irradiated BL samples. This observation supports the assumption that the mechanism of the formation of total DHdThd is the same between the γ- and the EB-irradiated BL samples. The total DHdThd is produced from dThd in aqueous solution by the indirect effects of irradiation. 22–31 The efficiency of total DHdThd formation may depend on the amount of H2O in the vicinity of the DNA. Therefore, water loss may reduce the
amount of total DHdThd generated by DNA irradiation. In our pilot study using fresh and dried red pepper samples, the efficiency of total DHdThd from fresh samples was ~2X greater than that of dried samples when both were irradiated under the same conditions (details will be published elsewhere). The isomer ratio was almost identical to that of the commercially obtained total DHdThd standard. Thus, the quantitation of total DHdThd was possible without a complicated process by LC–MS/MS analysis.

The dose–response curves of Liver I–III irradiated by EB and γ-rays nearly overlapped. Both the EB at 10 MeV and the γ-ray applied in the present study have low linear energy transfer and are deeply penetrating. Therefore, the efficiency of total DHdThd formation could depend mainly on the radiation dose. First, DNA is found in all BL samples and its content is similar for samples derived from different animals. Second, total DHdThd forms from the indirect effects of dThd irradiation.

The TL intensity increase and the PSL signal counts may decrease during the storage and heat treatment.32 Certain radicals detected by ESR may be stable enough to distinguish samples even 12 months after irradiation.33 Many radicals rapidly decay initially then gradually decay thereafter. Radical decay is influenced by heat treatment and humidity.33,34 ACB content in irradiated samples may also change during storage and heat treatment.35,36 However, total DHdThd/dThd was stable in samples stored at −20 °C for 4 months then heat-treated. MHLW reported that the core temperature of foods at risk of contamination with pathogens including EHEC must be maintained at 75 °C for ≥1 min. In the present study, irradiated Liver III was heat-treated in a microwave oven such that the aforementioned MHLW criteria were met. However, the DNA yield and the amount of nucleosides derived from the DNA were reduced by ≤50% compared to the unheated samples. DNA yield may have been caused by DNA damage, protein denaturation, inhibition of DNA extraction, and chloroform treatment. The release of nucleosides from DNA may be impeded by DNA damage and impurities formed during heat treatment. However, both total DHdThd and dThd decreased to the same degree in the heat-treated samples, so the total DHdThd/dThd was unchanged. The stability of this ratio in test solutions is indicative of the robustness of this method.

The sample irradiation dose could be estimated by comparing the total DHdThd/dThd with that of another sample. The irradiation dose was reasonably estimated from the equation derived from the dose–response curve of EB-irradiated Liver I. In the high irradiation ranges (4.67–7.62 kGy), the irradiation dose could be estimated from the dose–response curve of another irradiated sample to within the ratio of 0.74–1.30 to the actual dose. The average ± SEM of the 10 ratios (EB or γ irradiated BL samples, Liver I, Liver II, and Liver III including Liver III stored and Liver III heated) at the planned irradiation dose of 5 and 8 kGy corresponding to high irradiation ranges were 0.88 ± 0.03 and 1.03 ± 0.05, respectively. Meanwhile, the estimated doses varied widely at the low irradiation range (0.25–1.93 kGy) because of the slightly concave shape of the dose–response curves. Moreover, this estimate was virtually unaffected by differences in the irradiation source, storage period, and heat treatment and is a decided advantage of this method. Only a few reports that could estimate the irradiation dose of the sample can be found.33–36 With certain exceptions published by the World Health Organization (WHO), the maximum recommended irradiation dose is set to 10 kGy.38 The approved maximum irradiation dose for frozen meat product is 7.0 kGy in the U.S. However, no suitable method of estimating the irradiation dose has been established. The methods of determining irradiation history established by CEN only identify irradiated samples. In contrast, the new method proposed herein would facilitate the direct measurement of irradiation doses in foods. This method could be applicable to the examination of irradiation dose of foods with the approved maximum irradiation around 4.5–7.5 kGy to control the limit of irradiation dose properly. Ideally, the ratio of the estimated dose to the actual dose could be closer to 1.00. However, the difference of ratio to 1.00 might continuously persist as the actual irradiation dose was determined by a well-controlled radioclinical film dosimetry and total DHdThd/dThd in the BL samples were determined by experimental procedures. To decrease the difference in the ratio to 1.00, the reduction of the experimental variation will be required. For instance, using the surrogate of total DHdThd may be effective; studies for improving the experimental procedure using the surrogate of total DHdThd are ongoing in our laboratory.

## CONCLUSIONS
Total DHdThd/dThd may be a robust, radiospecific, and dose-dependent marker of BL irradiation history. The total DHdThd/dThd dose–response curve was not affected by differences in individual animals, irradiation source, sample storage period, or heat treatment. In view of its robustness, then, the dose–response curves of previous samples could be used to estimate the irradiation doses of current ones. This method may be the first which enables the direct determination of irradiation doses in foods.

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## ABBREVIATIONS

ACBs, 2-alklycyclobutanones; ALP, alkaline phosphatase; BL, beef liver; CEN, Le Comité Européen de Normalisation; DHdThd, 5,6-dihydrothymidine; dThd, 2′-deoxythymidine; EB, electron beam; EHEC, enterohemorrhagic *Escherichia coli*; ESR, electron spin resonance; LC–MS/MS, liquid chromatography–coupled to tandem mass spectrometry; MHLW, Ministry of Health, Labour, and Welfare; NaI, sodium iodide; 8-oxo-dGuo, 8-oxo-2′-deoxyguanosine; PP, polypropylene; PVDC, poly(vinylidene chloride); PSL, photostimulated luminescence; SD, standard deviation; SEM, standard error of mean;
SVPD, snake venom phosphodiesterase I; TL, thermoluminescence; WHO, World Health Organization

# REFERENCES

(1) Arvanitoyannis, I. S.; Stratakos, A.; Mente, E. Impact of irradiation on fish and seafood shelf life: a comprehensive review of applications and irradiation detection. Crit. Rev. Food Sci. Nutr. 2009, 49, 68–112.

(2) Arvanitoyannis, I. S.; Stratakos, A.; Tsarouhas, P. Irradiation applications in vegetables and fruits: a review. Crit. Rev. Food Sci. Nutr. 2009, 49, 427–462.

(3) Dempster, J. F. Radiation preservation of meat and meat products: A review. Meat Sci. 1985, 12, 61–89.

(4) IAEA. Food & Environmental Protection Newsletter, Vol. 9, 2006. http://www-naewb.iaea.org/nafa/lep/public/lep-nd-9-2.pdf (accessed June 14, 2019).

(5) Kume, T.; Furuta, M.; Todoriki, S.; Uenoyama, N.; Kobayashi, Y. Status of food irradiation in the world. Radiat. Phys. Chem. 2009, 78, 222–226.

(6) U.S. Food and Drug Administration. Ionizing radiation for the treatment of food, Code of Federal Regulations, Title 21, Vol. 3, 2016. http://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cffcr/ CFRSearch.cfm?fmr=179.26 (accessed June 14, 2019).

(7) Watahiki, M.; Isobe, J.; Kimata, K.; Shima, T.; Kanatani, J.; Shimizu, N.; Nagata, A.; Kawakami, K.; Yamada, M.; Izumiya, H.; Iyoda, S.; Morita-Ishihara, T.; Mitobe, J.; Terajima, J.; Ohnishi, M.; Sata, T. Characterization of enterohemorrhagic Escherichia coli O111 and O157 strains isolated from outbreak patients in Japan. J. Clin. Microbiol. 2014, 52, 2757–63.

(8) National Institute of Infectious Diseases. Enterohemorrhagic Escherichia coli infection in Japan as of April 2012; IASSR, 2012; Vol. 33, pp 115–116. https://www.mhlw.go.jp/mhlw/k Sang, A. G.; Madugundo, G. S.; Garcia, G.; Wagner, J. R.; Sanche, L. Thymidine decomposition induced by low-energy electrons and soft X rays under N2 and O2 atmospheres. Radiat. Res. 2014, 181, 629–40.

(10) Foodstuffs - Detection of irradiated food containing fat - Gas chromatographic/mass spectrometric analysis of 2-alkylcyclobutanones, EN1785; European Committee for Standardization: Brussels, 2003.

(11) Chen, S.; Morita, Y.; Saito, K.; Kameya, H.; Nakajima, M.; Todoriki, S. Identification of irradiated prawn (Penaeus monodon ) using thermoluminescence and 2-alkylcyclobutanone analyses. J. Agric. Food Chem. 2011, 59, 78–84.

(12) Fukui, N.; Takatori, S.; Kitagawa, Y.; Okihashi, M.; Ishikawa, E.; Fujiyama, T.; Kajimura, K.; Furuta, M.; Obana, H. Determination of irradiation histories of raw beef livers using liquid chromatography-tandem mass spectrometry of 5,6-dihydroxytrindole. Food Chem. 2017, 216, 186–193.

(13) Sharpatyi, V. A.; Cadet, J.; Teoule, R. Final products obtained from the gamma radiolysis of frozen aqueous solutions of thymidine. Int. J. Radiat. Biol. Relat. Stud. Phys., Chem. Med. 1978, 33, 419–423.

(14) Tyree, A. L.; Bonwick, G. A.; Smith, C. J.; Coleman, R. C.; Beaumont, P. C.; Williams, J. H. H. Detection of irradiated food by immunoassay — development and optimization of an ELISA for dihydroxymyidine in irradiated prawns. Int. J. Food Sci. Technol. 2004, 39, 533–540.

(15) Fukui, N.; Takatori, S.; Kitagawa, Y.; Fujirwara, T.; Ishikawa, E.; Fujiyama, T.; Kajimura, K.; Furuta, M.; Obana, H. Rapid and reliable method for determining irradiation histories of ground beef and prawns by measuring 5,6-dihydroxytrindole. Food Chem. 2017, 65, 9342–9352.

(16) Foodstuffs - Thermoluminescence detection of irradiated food from which silicate minerals can be isolated, EN1788; European Committee for Standardization: Brussels, 2001.

(17) Foodstuffs - Detection of irradiated food using photostimulated luminescence, EN13751; European Committee for Standardization: Brussels, 2009.

(18) Jin, Q. W.; Ahn, J. J.; Kim, G. R.; Kim, D. G.; Kwon, J. H. Luminescence characteristics for identifying irradiated black soybeans. Int. J. Radiat. Biol. 2010, 86, 842–847.

(19) Kim, H. Y.; Ahn, J. J.; Shahbaz, H. M.; Park, K. H.; Kwon, J. H. Physical-, chemical-, and microbiological-based identification of electron beam- and gamma-irradiated frozen crushed garlic. J. Agric. Food Chem. 2014, 62, 7920–7926.

(20) Song, B. S.; Kim, B. K.; Yoon, Y. M.; Jung, K.; Park, J. H.; Kim, J. K.; Kim, C. T.; Lee, Y.; Kim, D. H.; Ryu, S. R. Identification of red pepper powder irradiated with different types of radiation using luminescence methods: A comparative study. Food Chem. 2016, 200, 293–300.

(21) Gadgil, P.; Hachmeister, K. A.; Smith, J. S.; Kropf, D. H. 2-alkylcyclobutanones as irradiation dose indicators in irradiated ground beef patties. J. Agric. Food Chem. 2002, 50, 5746–5750.

(22) Foodstuffs - Detection of irradiated food containing bone - Method by ESR spectroscopy, EN1786; European Committee for Standardization: Brussels, 1996.

(23) Desrosiers, M. F. Assessing radiation dose to food. Nature 1990, 345, 485.

(24) Dodd, N. J.; Lea, J. S.; Swallow, A. J. The ESR detection of irradiated food. Int. J. Radiat. Appl. Instrum., Part A 1989, 40, 1211–1214.

(25) Wang, L.; Hirayasu, K.; Ishizawa, M.; Kobayashi, Y. Purification of genomic DNA from human whole blood by isopropanol-fractionation with concentrated NaI and SDS. Nucleic Acids Res. 1994, 22, 1774–1775.

(26) Cadet, J.; Wagner, J. R.; Shafirovich, V.; Geacintov, N. Evidence of radiation on fish and seafood shelf life: a comprehensive review of applications and irradiation detection. Crit. Rev. Food Sci. Nutr. 2019, 4, 12325–12332.

(27) Hua, Y.; Wainhaus, S. B.; Yang, Y.; Shen, L.; Xiong, Y.; Xu, X.; Zhang, F.; Bolton, J. L.; van Bremen, R. B. Comparison of negative and positive ion electrospray tandem mass spectrometry for the liquid chromatography tandem mass spectrometry analysis of oxidized deoxyureidoxesides. J. Am. Soc. Mass Spectrom. 2001, 12, 80–87.

(28) Alizadeh, E.; Sanz, A. G.; Madugundo, G. S.; Garcia, G.; Wagner, J. R.; Sanche, L. Thymidine decomposition induced by low-energy electrons and soft X rays under N2 and O2 atmospheres. Radiat. Res. 2014, 181, 629–40.

(29) Dizdaroglu, M.; Jaruga, P. Mechanisms of free radical-induced damage to DNA. Free Radic. Res. 2012, 46, 382–419.

(30) Sharma, K. K.; Swarts, S. G.; Bernhard, W. A. Mechanisms of direct radiation damage to DNA: the effect of base sequence on base end products. J. Phys. Chem. B 2011, 115, 4843–55.

(31) Shaw, A. A.; Voituriez, L.; Cadet, J.; Gregoli, S.; Szymons, M. C. R. Identification of the products resulting from the direct effects of γ-radiation on thymidine. J. Chem. Soc, Perkin Trans. 2 1988, 1303–1307.

(32) Lee, J.; Kausar, T.; Kim, B. K.; Kwon, J. H. Detection of gamma-irradiated sesame seeds before and after roasting by analyzing photostimulated luminescence, thermoluminescence, and electron spin resonance. J. Agric. Food Chem. 2008, 56, 7184–7188.

(33) Stachowicz, W.; Burlinska, G.; Michalik, J.; Dziezidz-Goclawaska, A.; Ostrowski, K. EPR Spectroscopy for the Detection of Foods Treated with Ionizing Radiation. In Detection Methods for Irradiated Foods, Current Status; McMurry, H. C.; Stewart, E. M.; Gray, R.; Pearce, J., Eds.; The Royal Society of Chemistry: Cambridge, U.K., 1996; pp 23–32.

(34) Foodstuffs - Detection of irradiated food containing cellulose by ESR spectroscopy, EN1787; European Committee for Standardization: Brussels, 2000.

(35) Kwon, J. H.; Akram, K.; Nam, K. C.; Lee, E. J.; Ahn, D. U. Evaluation of radiation-induced compounds in irradiated raw or cooked chicken meat during storage. Poult. Sci. 2011, 90, 2578–2583.

(36) Obana, H.; Furuta, M.; Tanaka, Y. Detection of irradiated meat, fish and their products by measuring 2-alkylcyclobutanones levels after frozen storage. Shokuhin Eiseigaku Zasshi 2007, 48, 203–6.

(37) Gadgil, P.; Smith, J. S.; Hachmeister, K. A.; Kropf, D. H. Evaluation of 2-dodecylcyclobutanone as an irradiation dose indicator
in fresh irradiated ground beef. *J. Agric. Food Chem.* **2005**, *53*, 1890–1893.

(38) WHO. *High-dose irradiation: wholesomeness of food irradiated with doses above 10 kGy*, Report of a Joint FAO/IAEA/WHO Study Group, Geneva, Technical Report Series, 890, 1999. https://apps.who.int/iris/handle/10665/42203 (accessed June 14, 2019).