# Effects of Alpha-Tocopherol on the Formation of Lactones in Beef Rendered Fat

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***Abstract*— The formations of aliphatic lactones were studied on high marbled beef. A standard-addition method revealed the concentration of gamma-octalactone, gamma-nonalactone, delta-decalactone, delta-dodecalactone and delta-tetradecalactone in rendered beef fat. All lactones were increased during 7 days storage, and the highest incremental increase of 11.7-fold higher than day 0 was gamma-nanalactone, with a concentration of 51.4 ppb. The highest concentration was observed for delta-tetradecalactone, which was 297.8 ppb and 5.9-fold higher than day 0. Addition of alpha-tocopherol inhibited the formation of gamma-octalactone and gamma-nonalactone, but the effect was ambiguous for delta-lactones. Matrix effect changes increased volatility during storage, and was observed for all lactones except gamma-octalactone, but quantitative analysis revealed increases in the intensity of lactones in headspace during storage were due to matrix changes and lactones production.**

*Keywords*— **Lactones, beef, alpha-tocopherol.**

## I. INTRODUCTION

Japanese Black Cattle, known as Wagyu, are famous for marbled beef [1]. The average fat content was about 34%, when recently measured [2]. The reason for producing high marbled beef is not only tenderness but also its sweet aroma, which are recognized to be due to lactones [3]. Lactones as a desirable flavor have been studied in many foods, including dairy products, meat, and beverages, though early reports from the 1950s considered lactones an undesirable flavor in whole milk powder [4]. In beef production, lactones play a role in producing the desirable deep fat-fried flavor and masking of grassy flavors [5]. Although it is not clear whether lactones are produced during storage and/or cooking, they were detected at higher levels in fat from animals fed grain-based diets compared to grass-fed animals [6]. In contrast, headspace analysis using solid-phase microextraction (SPME) fiber suggested the production of some lactones, or possibly precursors, were inhibited by the addition of alpha-tocopherol (alpha-Toc) [7]. However, head-space analysis does not represent lactones concentration in the sample because of differences in the extraction efficiency between the phases [8]. The present study was undertaken to measure the concentration of lactones in samples, using a standard addition method for SPME-GCMS analysis, and to clarify the effect of alpha-Toc on the formation of lactones.

## II. MATERIALS and METHODS

### *A. Sample preparation*

Frozen beef from Japanese Black Cattle (Wagyu) was used. Vacuum packed *M.longissimusthoracis*, with 23.4% fat, was thawed at 2 ºC over night. The core of the muscle was minced, and divided into three groups with different alpha-Toc levels, according to our previous paper [7]. Alpha-Toc concentrations were determined by HPLC analyses [9]. Samples were mixed with chlorotetracycline-hydrochloride saline solution, to prevent microorganism growth. Samples from all of the groups were stored at 2 ºC for 7 days.

### *B. Lipid peroxidation analysis*

Lipid peroxidation was evaluated before and after storage by the production of thiobarbituric acid reactive substances (TBARS) using the method of Kikugawa and others [10].

### *C. Extraction procedure and GCMS analysis*

Volatiles were extracted using the method described by us previously [7]. Rendered fat (0.1 g) was removed to a glass crimp-top vial, and 2.0 $\mu$L of 2, 4, 6-trichloroanisole (10 $\mu$g/mL in methanol) was added as an internal standard (IS). Commercially available aliphatic lactones, detected in our previous paper [7]: gamma-octalactone (gamm-C8), gamma-nonalactone

(gamma-C9), delta-decalactone (delta-C10), delta-dodecalactone (delta-C12) and delta-tetradecalactone (delta-C14), were purchased and diluted with pure methanol for standard solutions. For calibration curve construction, samples with IS in vials were spiked with the standard solutions.

The vials were heated for 10 min at 100 ºC using a block bath, and the volatiles were collected by divinylbenzene/carboxen/polydimethylsiloxane fiber as SPME (Supelco, PA., U.S.A.), and analyzed using a GC-MS-QP2010 instrument (Shimadzu Co., Ltd., Kyoto, Japan) equipped with a DB-17MS (30 m length, 0.32 mm inside diameter, and 0.25 $\mu$m film thickness; Agilent Technologies Inc., CA., U.S.A.). The analytical conditions and peak identification were the same as described in our previous report [7]. These analyses were repeated three times and the data were submitted for statistical processing.

*D. Statistical analysis*

The whole set of data was submitted to analysis of variance, and the differences between groups were evaluated by Tukey's test using SAS 9.2 TS Level 2M0 (SAS Inst. Inc., Cary, N.C., U.S.A.).

## III. RESULTS

*A. Effect of alpha-Toc as an antioxidant*

The concentrations of alpha-Toc were 2.9, 10.8 and 28.8 ppm in the NE, ME and HE-group, respectively. Addition of alpha-Toc clearly inhibited lipid peroxidation during 7 days storage (Table 1).

**Table 1 Changes in lipid peroxidation after storage**

| Groups | Before storage | After storage | |
|---|---|---|---|
| NE | 0.059 ± 0.010 | 0.233 ± 0.032 | a |
| ME | 0.059 ± 0.006 | 0.184 ± 0.009 | b |
| HE | 0.076 ± 0.012 | 0.142 ± 0.004 | b |

Groups of NE, ME and HE containing different levels of alpha-Toc; 2.9, 10.8 and 28.8 ppm, respectively. Different letters (a, b) indicate significant difference between the groups ($P$ <0.05).

There were no differences between the groups before storage, and significant effects ($P$=0.0035) were observed after storage, where the higher alpha-Toc levels led to lower lipid peroxidation. Significant differences were found between NE and ME, and between NE and HE ($P$<0.05).

*B. Accuracy and matrix effects*

The standard addition method was used to determine the concentration of lactones in rendered fat. Table 2a shows the slopes and correlation coefficients of the regression curves.

**Table 2a Slopes and correlation coefficients**

| Lactones | Groups | Before storage | After storage |
|---|---|---|---|
| γ-C8 | NE | 30.6 ± 6.0 (1.00) | 31.6 ± 3.1 (0.97) |
| | ME | 32.3 ± 1.1 (1.00) | 32.0 ± 1.6 (0.99) |
| | HE | 32.8 ± 1.9 (0.99) | 32.9 ± 1.3 (1.00) |
| γ-C9 | NE | 26.3 ± 3.5 (1.00) | 34.9 ± 1.4 (0.98) |
| | ME | 26.3 ± 1.8 (1.00) | 33.8 ± 0.9 (1.00) |
| | HE | 29.9 ± 1.7 (1.00) | 35.6 ± 1.7 (1.00) |
| δ-C10 | NE | 9.3 ± 0.4 (0.98) | 15.1 ± 0.6 (0.98) |
| | ME | 8.9 ± 0.8 (1.00) | 13.7 ± 2.3 (0.99) |
| | HE | 10.2 ± 0.8 (0.99) | 14.8 ± 0.7 (0.99) |
| δ-C12 | NE | 4.7 ± 0.5 (0.95) | 9.0 ± 1.9 (0.97) |
| | ME | 3.9 ± 0.0 (0.97) | 7.8 ± 2.4 (0.96) |
| | HE | 4.6 ± 0.6 (0.96) | 8.5 ± 0.4 (0.96) |
| δ-C14 | NE | 2.3 ± 1.0 (0.94) | 6.1 ± 2.3 (0.97) |
| | ME | 2.1 ± 0.0 (0.93) | 5.5 ± 2.1 (0.94) |
| | HE | 2.3 ± 0.6 (0.89) | 6.2 ± 0.7 (0.96) |

Correlation coefficients are showed in parentheses. Different letters (a, b) indicate significant difference between the groups ($P$ <0.05).

**Table 2b Statistical analysis (*P*-values)**

| Factor | Lactones | | | | |
|---|---|---|---|---|---|
| | γ-C8 | γ-C9 | δ-C10 | δ-C12 | δ-C14 |
| Storage effect | 0.861 | **<0.0001** | **<0.0001** | **<0.0001** | **<0.0001** |
| α-Toc treatment | 0.618 | 0.101 | 0.258 | 0.425 | 0.783 |
| Interaction | 0.940 | 0.501 | 0.650 | 0.938 | 0.955 |

The slope was determined from the following linear regression curve**:**

$Y = ax+b$

Where Y = (peak area of the analyte) x 1000 / (peak area of IS)

x = concentration (ppb) of standard solutions in the sample

High linear correlations ($r^2$>0.97) were observed for the lower molecular lactones; gamma-C8, gamma-C9 and delta-C10. In contrast, lower correlations ($r^2$<0.95) were observed for delta-C12 and C14 in some groups, and the slope of these lactones, including delta-C10, were lower than those of gamma-C8 and gamma-C9. Table 2b shows the results of the statistical analysis. Slopes were significantly increased ($P$<0.0001) during storage, except for gamma-C8.

*C. Quantification*

Lactone concentrations in rendered fat, before and after storage, are shown in table 3.

**Table 3 Concentration of lactones in rendered fat (ppb)**

| Lactones | Groups | Before storage | After storage |
|---|---|---|---|
| γ-C8 | NE | 4.7 ± 1.0 | 35.0 ± 8.1 a |
| | ME | 5.8 ± 4.1 | 24.5 ± 5.7 ab |
| | HE | 7.5 ± 2.4 | 18.2 ± 1.6 b |
| γ-C9 | NE | 4.4 ± 1.6 | 51.4 ± 8.0 a |
| | ME | 8.8 ± 3.2 | 35.7 ± 0.9 b |
| | HE | 5.9 ± 2.6 | 29.5 ± 4.5 b |
| δ-C10 | NE | 13.7 ± 6.0 | 24.8 ± 2.7 |
| | ME | 30.6 ± 6.0 | 31.6 ± 8.2 |
| | HE | 18.4 ± 8.9 | 35.5 ± 5.2 |
| δ-C12 | NE | 46.2 ± 27.3 | 94.4 ± 7.1 |
| | ME | 98.3 ± 4.2 | 121.2 ± 37.1 |
| | HE | 65.1 ± 20.0 | 141.7 ± 11.2 |
| δ-C14 | NE | 50.7 ± 21.6 | 297.8 ± 108.5 |
| | ME | 227.8 ± 42.2 | 346.9 ± 132.4 |
| | HE | 159.8 ± 70.6 | 415.8 ± 70.5 |

Groups of NE, ME and HE containing different levels of alpha-Toc; 2.9, 10.8 and 28.8 ppm, respectively. Different letters (a, b) indicate significant difference between the groups ($P$ <0.05).

The concentrations of all lactones were increased during storage. The concentrations of gamma-lactones were less than 10.0 ppb before storage, which is clearly lower than those of the delta-lactones. The gamma-lactones were significantly increased ($P$<0.0001) during storage, and the highest incremental increase was observed in the NE-group: 4.7 to 35.0 ppb for gamma-C8 (7.4 fold higher than day 0), and 4.4 to 51.4 ppb for gamma-C9 (11.7-fold higher than day 0). The lowest increase was observed in the highest alpha-Toc group (HE-group).

In contrast, the presences of some delta-lactones were observed before storage. The increases during storage were statistically significant ($P$<0.05) for delta-C10, delta-C12 and delta-C14, but these incremental increases were smaller compared with the increases in gamma-C8 and gamma–C9, and were ambiguous in some groups. The effects of alpha-Toc were not clearly detected in delta-C10, delta-C12 and delta-C14 ($P$>0.05). The highest concentration was observed in delta-C14 at day 7: 297.8, 346.9 and 415.8 ppb of the NE, ME and HE-group, respectively.

## IV. DISCUSSION

Increasing alpha-Toc from 2.9 to 28.8 ppm led to a linear decrease of the TBARS value (Table 1). Mitsumoto et al. [11] reported approximately 3.5 ppm of alpha-Toc was enough to produce an effective lowering of the TBA value in Holstein meat, and 2.4 ppm was enough for Wagyu beef [12]. Elevations of greater than 10 ppm by diet may be possible when higher marbling beef is used, however, elevations of 28.8 ppm, observed in the HE-group, are not realistic. The HE-group was used in the present study to clarify the effect of alpha-Toc on lactones formation.

Linear regression curves were constructed to determine the concentration of lactones. High linearity was observed for gamma-C8, gamma-C9 and delta-C10; the linearity was lower in delta-C12 and delta-C14 in some groups. The slopes of delta-C10, delta-C12 and delta-C14 were lower than gamma-C8 and gamma-C9. These differences probably affected the concentration calculations, since the standard deviations (SD) were larger in delta-C12 and delta-C14 compared with gamma-C9 and gamma-C8 (Table 3). In the present study, we did not use a salting-out agent, such as $Na_2SO_4$, to increase volatility because one of our aims was to show the change of matrix effects. When more accurate analyses are required, a proper salting-out agent should be used.

The slope changes after storage indicated the distribution constant of sample/headspace were probably changed by the production of chemicals during storage, suggesting the volatility of the lactones was increased.

We have reported on the intensity of lactones in headspace [7], but the intensity is affected by the partition equilibrium between sample matrix and headspace. Quantitative analysis of samples was performed in the present study, and we suggest the resulting increase in the intensity of lactones during storage is due to matrix changes and lactone production. The pattern in production of gamma-C8 and gamma-C9 is the same as the pattern in the development of peroxidation (Table 1), indicating the formation of gamma-lactones, which are key factors in the Wagyu flavor, would be mainly processed during storage by oxidation.

Delta-lactone formation was not completely controlled by alpha-Toc, indicating another pathway may exist. Delta-C10, delta-C12 and delta-C14 were already present at 10 to 100 ppb before storage, which is higher than gamma-C8 and gamma-C9 (Table 3). Stark et al. [13] proposed the process of lactone formation in milk, which undergoes hydration and beta-oxidation in live animals. This suggests delta-lactones in beef fat may be partly produced in live animals.

Usually, the higher the levels of alpha-Toc, the better the beef is, however, how the beef will be consumed must be considered with the levels of alpha-Toc.

## V. CONCLUSIONS

The concentration of gamma-C8- and gamma-C9-lactone in fat decreased in a linear manner with increased alpha-Toc concentration. These observations suggested that an excessive increase in alpha-Toc could reduce the formation of positive flavor components in marbled beef.

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