

## Liposoluble Anti Oxidative Components in Japanese Traditional Fermented Food “Amazake” Made from Brown Rice

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### Abstract

Amazake is nutrient-rich and physiologically active Japanese traditional food made by fermenting steamed rice with 'koji' mold. In this study, vitamin E species were determined as liposoluble antioxidants in amazake and changes in their compositions were examined during fermentation. Six vitamin E species were detected in brown rice amazake (BRA), and the total vitamin E content in BRA was six-fold higher than that in white rice amazake. This was consistent with the high radical scavenging capacity of BRA, which corresponded with the total levels of vitamin E and ferulic acid (FA). Although vitamin E levels in brown rice powder decreased during presaccharification, their levels were almost constant throughout saccharification and remained high in the final BRA product, suggesting the liposoluble antioxidants were stable under amazake preparation conditions. 4-Vinylguaiacol (4-VG) was found in amazake, and its content markedly increased during BRA manufacturing. Rice koji contained high levels of 4-VG produced from FA by the biological action of koji. The liposoluble antioxidative activities in BRA were due to vitamin E and 4-VG, in addition to FA, a phenolic compound thought to be the major radical scavenger. These observations suggested the superiority of traditional fermentation using koji and the advantage of BRA containing liposoluble antioxidants, such as vitamin E and FA.

**Keywords:** Amazake; Brown rice; Saccharification; Vitamin E; Ferulic acid; 4-Vinylguaiacol

### Introduction

Brown (unpolished) rice contains more nutrients, such as fat, dietary fiber, liposoluble vitamins, and water-soluble vitamins, than white (polished) rice. As most of these nutrients are contained in the rice bran [1], liposoluble nutrients are found in rice bran essential oil, which contains various kinds of physiologically active compounds, such as several phytosterols,  $\gamma$ -oryzanol, and vitamin E compounds, including tocopherols and tocotrienols. In addition to vitamin E action, tocopherols are known as antioxidants due to their chromanol ring [2,3], which affords an anti-inflammatory effect that reduces the risk of cardiovascular disease [4,5]. Recently, tocotrienols have been reported to show unique physiological characteristics, such as hypocholesterolemic, anticancer, antidiabetic, neuroprotective, hepatoprotective, and nephron-protective effects [6-8]. Rice bran oil containing vitamin E nutrients has been shown to significantly suppress hyperlipidemic responses in diabetic rats through the hypocholesterolemic effect of these ingredients [9,10]. Although rice bran oil is a promising health promoting food, it is not popular in Asian countries because the necessary removal of a large amount of wax contaminant makes it expensive. Although eating steamed brown rice is straightforward, it also has several problems, such as bad texture, poor flavor, and poor digestion.

Fermenting foods improves their nutrient compositions and has therefore attracted much attention for its great potential for health promotion [11]. In Japan, koji mold has been utilized to make various kinds of traditional Japanese fermented foods. Koji mold is a microbial flora consisting mostly of *Aspergillus* sp., especially *Aspergillus oryzae*

(*A. oryzae*). *A. oryzae* is nonpathogenic in human beings [12] and designated as the Japanese national microorganism because of its wide use in Japanese food. Koji is made by the solid-state fermentation of koji mold on whole grains, such as rice, soy beans, and wheat. Rice koji is also utilized in manufacturing, such as in the production of Japanese rice wine (sake in Japanese) and amazake. Amazake is a viscous turbid sweet drink made by the fermentation of rice by rice koji, and is used as a sugar substitute and nutritious food. Various kinds of amazake are now commercially produced in Japan, including white rice amazake (WRA) and sake cake amazake (SCA). Sake cake is a solid residue obtained by refining sake, which is made of highly polished rice. Several types of brown rice amazake (BRA) are also on the market. BRA has attracted attention as a macrobiotic functional food with possible physiological activity. BRA is an effective chemo preventive agent against inflammation-related gastric [13] and colorectal [14] carcinogenesis, and reduces expression of inflammation-related genes, such as TNF- $\alpha$ , Mac-1, CCL 3, and CXCL 2 [15]. Changes in nutrition during food processing can directly affect the food function, though this has not been sufficiently examined. Numerous hydrophobic vitamins are present in rice bran, meaning that hydrophobic antioxidant components are expected to be involved in the physiological effects of BRA. Therefore, we previously investigated the antioxidant activity of BRA in vitro and found that the antioxidant activity of BRA was higher than those of WRA and SCA [16-20]. Furthermore, there was a lack of correlation between the radical scavenging ability of BRA and the polyphenol content, which indicated the contribution of other uncharacterized antioxidants. In this context, we now turned our focus to the liposoluble antioxidant components in amazake. Herein, we report the quantitative analysis of vitamin E compositions and ferulic acid (FA) derivatives to determine the hydrophobic chemical substances contributing to antioxidative activity in amazake [21-29]. As the temperature of amazake production is high, and hot amazake is

usually drunk in winter, the thermal stability of vitamin E was also tested under experimental conditions to mimic amazake production.

## Materials and Methods

### General

BRA, WRA, brown rice powder, and dry brown rice koji were supplied by Yamato Soy Sauce & Miso (Kanazawa, Japan). A second BRA sample (BRA2) was purchased from Ayumasamune-shuzo (Myoko, Japan), and a second WRA sample (WRA2) was obtained from Fukumitsuya Sake Brewery (Kanazawa, Japan). Two sake cake amazake samples, SCA and SCA2, were purchased from Morinaga (Tokyo, Japan) and Melodian (Osaka, Japan), respectively. Dry white rice koji was purchased from Misuzu Shokuhin (Nakatsugawa, Japan). Steamed brown rice was purchased from Kojima foods (Nagoya, Japan). Multi-enzyme complex was made by Amano Enzyme (Nagoya, Japan). DL- $\alpha$ -tocopherol, ferulic acid, and 4-vinylguaiacol (4-VG) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).  $\beta$ -Tocopherol and  $\delta$ -tocotrienol were purchased from Santa Cruz Biotechnology (Santa Cruz, USA).  $\gamma$ -Tocopherol and  $\delta$ -tocopherol were purchased from Sigma-Aldrich (St. Louis, USA).  $\alpha$ -Tocotrienol,  $\beta$ -tocotrienol, and  $\gamma$ -tocotrienol were purchased from LKT Laboratories (St. Paul, USA). All other chemicals used in this research are listed in the supplementary information. Ultrapure water was prepared using a PWU-100 purification system (Advantec, Tokyo, Japan). Centrifugation was performed using an LC-120 centrifuge (TOMY, Japan). Normal phase HPLC (NP-HPLC) analysis was performed using an HPLC system equipped with an L-6000 pump and a 655-A-52 column oven (Hitachi High-Technologies, Tokyo, Japan), a 2465 multi- $\lambda$  fluorescence detector (Waters, Milford, USA), and a C-R6A chart recorder (Shimadzu, Kyoto, Japan). Mightysil Si60 (5  $\mu$ m, 150-4.6 mm, Kanto Chemical Co. Inc., Tokyo, Japan) was used as the separation column stationary phase, with the temperature maintained at 30 °C. Reverse-phase HPLC (RP-HPLC) was performed using an HPLC system with a PU-2089 pump and MD-2018 detector (Jasco, Tokyo, Japan), and a Wakopak HandyODS column (4.6  $\times$  150 mm, Wako Pure Chemical Industry). UV-Vis absorption spectra were obtained using a V-550 spectrophotometer (Jasco).

### Fermentation of brown rice powder by koji mold

Distilled water (3.31 mL) and brown rice powder (1.690 g) containing 0.05 wt% multi-enzyme complex were placed in a 15-mL glass vial and sealed tightly with a screw cap. A total of nine of these samples was prepared. One vial of the nine was kept for extraction, and the remaining eight vials were heat treated as follows, with one vial kept for extraction after each step: (i) Heated at 45°C for 1 h, (ii) heated at 70°C for 1 h, and (iii) heated at 95°C for 1 h. After these heat treatment (presaccharification) processes, brown rice koji (1.335 g) was added to the remaining five vials and incubated at 57°C. A vial was removed from the incubator for extraction at 0, 6, 12, 18, and 24 h after starting incubation. This process resulted in nine differently prepared samples, namely, an untreated sample, three thermally treated samples (at 45°C, 70°C, and 95°C), and five saccharification samples (at 0, 6, 12, 18, and 24 h). Four replicates were prepared for each sample.

### Extraction from wet food sample

Extraction from each food sample was performed according to a previous procedure [17] with a small modification. Wet food sample

(5.00 g), NaCl solution (1.80 mL, 10 g/L water), pyrogallol solution (200  $\mu$ L, 6 g/L ethanol), and ethyl acetate (3.00 mL) were mixed in a 15-mL centrifuge tube and shaken vigorously. The mixture was then centrifuged at 6700  $\times$  g for 10 min and the separated upper organic layer was collected. This extraction process was repeated three times using ethyl acetate as the extraction solvent. The combined organic layers were concentrated and dried in vacuo. The residue was dissolved in hexane (1.00 mL) and filtered through a syringe filter (0.45  $\mu$ m) before HPLC analysis. The filtrate was also used for 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging analysis. Each food sample was shaken before extraction.

### NP-HPLC analysis and quantifying vitamin E and 4-VG

Vitamin E analysis was performed by NP-HPLC using an isocratic mobile phase (hexane/1,4-dioxane, 1000:25 v/v) at a flow rate of 2 mL/min. Using the correct eluent was important for separating  $\alpha$ -tocotrienol from 4-VG. Vitamin E and 4-VG were detected by their fluorescence excitation at 294 nm, and emission was recorded at 326 nm. Vitamin E and 4-VG peaks were assigned by comparison with standard samples. Vitamin E standard samples (0-100  $\mu$ M) and 4-VG standard sample (0-100  $\mu$ M) were constructed for quantification. The molar concentrations of vitamin E and 4-VG were converted into compound weight per dry weight of food ( $\mu$ g/g dry food) and/or mole per dry weight of food (nmol/g dry food). The dry weight of each food sample was measured after freeze-drying.

### RP-HPLC analysis and quantifying FA derivatives in food samples

Wet food sample (500 mg) was hydrolyzed in 4 N NaOH solutions (2 mL) at room temperature for 3 h [18]. The hydrolysate was neutralized by the addition of 4.2 N HCl solutions (2 mL) and then diluted with methanol (4 mL). After centrifugation to remove the debris, the syringe-filtered (0.45  $\mu$ m) solution was analyzed by RP-HPLC. Elution was performed using an isocratic mobile phase (water/methanol, 50:50 v/v) containing 0.1 vol% acetic acid at a flow rate of 0.6 mL/min. FA was detected by its absorbance at 300 nm. The FA concentration was determined using a calibration curve constructed using known concentrations (0-40  $\mu$ M) of authentic FA standard. After each measurement, the ODS column was washed with methanol (0.1 vol% acetic acid) to remove adsorbed lipid contaminant. Molar concentrations estimated using this method was converted into FA mole per dry weight of food (nmol/g dry food).

### DPPH radical scavenging capacity of the food extract

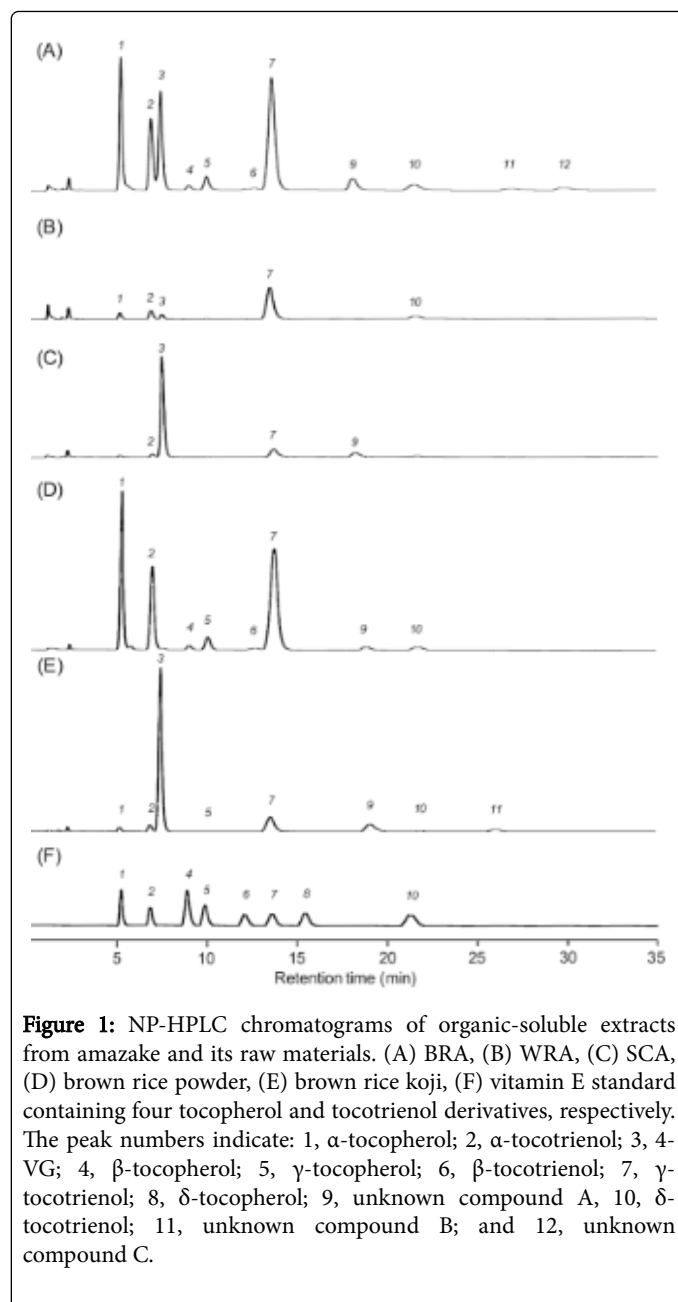
A DPPH stock solution was prepared in absolute ethanol on ice. DPPH solution (150  $\mu$ L), food extract dissolved in hexane, and hexane were mixed in a 1.5-mL sample tube to give a defined DPPH radical concentration ( $52 \pm 5$   $\mu$ M) and extract concentration (0-800  $\mu$ g/L). The reaction volume was set at 300  $\mu$ L. The absorption spectrum was measured after reacting for 15 min. The DPPH concentration, calculated from the absorbance at 515 nm, was plotted against extract concentration. A linear regression line was constructed from this plot. The number of moles of DPPH radical quenched by 1 g of dry weight of food was calculated from the x-axis intercept.

## Results and Discussion

### Determination of vitamin E contents in amazake and its raw materials

Figure 1 shows NP-HPLC chromatograms of organic-soluble extracts from amazake and its raw materials. BRA contained six vitamin E molecular species, while  $\delta$ -tocopherol and  $\beta$ -tocotrienol were not detected (Figure 1A). Four other peaks (peaks 3, 9, 11, and 12) were also detected, but were not attributable to known vitamin E molecular species (Figure 1A). WRA contained four vitamin E molecular species, namely  $\alpha$ -tocopherol and  $\alpha$ -,  $\gamma$ -, and  $\delta$ -tocotrienols (Figure 1B). SCA contained only  $\alpha$ - and  $\gamma$ -tocotrienols, but no tocopherols were detected (Figure 1C). The brown rice powder contained seven vitamin E molecular species, but  $\delta$ -tocopherol was not detected (Figure 1D). The extra peak (peak 9) was thought to be a fluorescent component derived from brown rice, and was not related to vitamin E or its derivatives. In brown rice koji, five vitamin E molecular species, namely  $\alpha$ - and  $\gamma$ -tocopherols and  $\alpha$ -,  $\gamma$ -, and  $\delta$ -tocotrienols, were detected (Figure 1E). In addition to these peaks, peak 3 was detected with a very high intensity, which suggested an uncharacterized fluorescent metabolite of koji mold (Figure 1E). To identify the fluorescent metabolite, the peak 3 fraction was purified from brown rice koji extract and its chemical structure was identified as 4-VG, as described in the supplementary information. A second BRA manufactured by a different vendor (BRA2) was analyzed in the same way. The detected components were essentially identical in both BRAs (Figure S1A), although the production processes and raw materials used by each vendor may be different. The level of 4-VG in BRA2 was much smaller than that in BRA (Figures 1A and S1A), which suggested that BRA2 had a shorter fermentation period and more highly sterilized conditions before packing, among other factors. A second WRA made by a different vendor (WRA2) contained three kinds of vitamin E molecular species (Figure S1B) with a slightly smaller variety than that of WRA. Vitamin E components in a second SCA provided by different vendor (SCA2) were almost same as SCA, while no 4-VG was detected (Figure S1C). It is because SCA2 was made of sake-cake, sugar, dextrin and salt.

Table 1 shows the vitamin E composition in amazake and its raw materials.  $\alpha$ -Tocopherol was the major tocopherol component ( $5.618 \pm 0.003 \mu\text{g/g}$ , 34 wt%) in BRA, while the other tocopherols had lower contents in the order  $\gamma$ -form ( $1.243 \pm 0.055 \mu\text{g/g}$ ) followed by  $\beta$ -form ( $0.285 \pm 0.022 \mu\text{g/g}$ ). Among the tocotrienols, the  $\gamma$ -form was the dominant species ( $5.886 \pm 0.185 \mu\text{g/g}$ , 35.7 wt%), followed by the  $\alpha$ -form ( $3.144 \pm 0.074 \mu\text{g/g}$ ) and the  $\delta$ -form ( $0.337 \pm 0.013 \mu\text{g/g}$ ). The total vitamin E content in BRA was calculated as  $16.51 \pm 0.32 \mu\text{g/g}$ . In BRA2, the vitamin E composition was similar to that of BRA, but the total vitamin E content was slightly lower ( $10.56 \pm 0.50 \mu\text{g/g}$ ). It was assumed that the breed of raw brown rice and/or the dilution ratio with water of BRA2 were different from those of BRA. The vitamin E compositions of WRA and WRA2 were different from that of BRA, in particular the  $\alpha$ -tocopherol level was markedly lower in WRA, with the content of  $\alpha$ -tocopherol in WRA ( $0.285 \pm 0.054 \mu\text{g/g}$ ) being only 5% that in BRA. The total vitamin E content was lower in both WRA ( $2.736 \pm 0.092 \mu\text{g/g}$ ) and WRA2 ( $1.759 \pm 0.040 \mu\text{g/g}$ ), compared with the levels in BRA. These results were consistent with the fact that brown rice contains vitamins E in the germ and bran [19], which should be removed during polishing when making white rice.



**Figure 1:** NP-HPLC chromatograms of organic-soluble extracts from amazake and its raw materials. (A) BRA, (B) WRA, (C) SCA, (D) brown rice powder, (E) brown rice koji, (F) vitamin E standard containing four tocopherol and tocotrienol derivatives, respectively. The peak numbers indicate: 1,  $\alpha$ -tocopherol; 2,  $\alpha$ -tocotrienol; 3, 4-VG; 4,  $\beta$ -tocopherol; 5,  $\gamma$ -tocopherol; 6,  $\beta$ -tocotrienol; 7,  $\gamma$ -tocotrienol; 8,  $\delta$ -tocopherol; 9, unknown compound A; 10,  $\delta$ -tocotrienol; 11, unknown compound B; and 12, unknown compound C.

Both SCA and SCA2 contained a small amount of  $\gamma$ -tocotrienol ( $0.443 \pm 0.029 \mu\text{g/g}$  and  $0.320 \pm 0.013 \mu\text{g/g}$ , respectively) as dominant species. The total vitamin E content of SCA was the smallest among the amazake samples. The vitamin E composition of brown rice powder, which is a raw material of BRA and thought to be the origin of vitamin E in BRA, was very similar to that of BRA, with a total vitamin E content ( $33.46 \pm 0.60 \mu\text{g/g}$ ) that was twice that of BRA. These results confirmed that the vitamin E components detected in BRA were mainly derived from brown rice powder with rice bran. In brown rice koji, which is the other raw material of BRA, the  $\gamma$ -tocotrienol content was very high ( $1.172 \pm 0.134 \mu\text{g/g}$ , 61.2 wt%), although the total vitamin E level was very low, at  $1.915 \pm 0.245 \mu\text{g/g}$ . Therefore, vitamin E in brown rice may be oxidized or thermally decomposed during the koji production process, and/or possibly metabolized by solid-state fermentation with *A. oryzae*. Fernandez-Orozco et al. reported that the

solid-state fermentation of soybean flour by *A. oryzae* changed its vitamin E composition [20]. Interestingly, the vitamin E composition of white rice koji was similar to that of brown rice koji (Figure S2), despite their different production processes and raw materials (brown and white rice). In gelatinized brown rice powder, the vitamin E composition was similar to that of the unheated brown rice powder (Figure S3), while the total amount of vitamin E was significantly decreased ( $5.778 \pm 1.147 \mu\text{g/g}$ ), which suggested the decomposition of vitamin E with heating. Adam et al. [21] and Kuppithayanant et al. [22] investigated the thermal decomposition of vitamin E in edible oil and found that vitamin E decomposed at a high temperature (above  $180^\circ\text{C}$ ). According to this observation, vitamin E may be stable during treatment in a boiling water bath. In contrast, Wanyo et al. reported the thermal decomposition of vitamin E at  $120^\circ\text{C}$  with hot-air treatment [23]. The thermal decomposition of vitamin E is thought to depend on the experimental conditions, including medium (such as

oxygen concentration, viscosity), shape of food (such as raw, ground, paste), and coexistence of antioxidant. Therefore, we tested the thermal decomposition of vitamin E in boiling water to mimic the amazake production process (Figure S4). An aqueous solution of  $\alpha$ -tocopherol ( $22 \mu\text{M}$ , 10 mL) containing 0.1 vol% ethanol was heated in a water bath for 1 h at  $95^\circ\text{C}$  and then extracted with hexane (1 mL). The upper organic layer was analyzed by NP-HPLC to determine the residual amount of  $\alpha$ -tocopherol. Approximately 60% of  $\alpha$ -tocopherol remained after 1 h at  $95^\circ\text{C}$ , which suggested that vitamin E underwent thermal and/or oxidizing decomposition during the gelatinization process. The vitamin E composition in steamed brown rice provided by Kojima foods was also determined. It contained only two kinds of tocotrienol ( $\alpha$ - and  $\gamma$ -tocotrienol) with very low contents ( $1.115 \pm 0.198 \mu\text{g/g}$  and  $2.053 \pm 0.401 \mu\text{g/g}$ , respectively). These results supported that amazake made from brown rice was a promising rice-derived food for supplementing vitamin E species efficiently.

Food sample	Tocopherol				Tocotrienol			
	Alpha	Beta	Gamma	Delta	Alpha	Beta	Gamma	Delta
<b>Amazake samples</b>								
BRA b	$5.618 \pm 0.003$	$0.285 \pm 0.022$	$1.243 \pm 0.055$	n.d. h	$3.144 \pm 0.074$	n.d. h	$5.886 \pm 0.185$	$0.337 \pm 0.013$
	-34.00%	-1.70%	-7.50%	0%	-19.00%	0%	-35.70%	-2.00%
BRA2 c	$2.359 \pm 0.194$	$0.182 \pm 0.032$	$0.872 \pm 0.086$	n.d. h	$1.971 \pm 0.102$	n.d. h	$4.847 \pm 0.114$	$0.329 \pm 0.009$
	-22.30%	-1.70%	-8.30%	0%	-18.70%	0%	-45.90%	-3.10%
WRA b	$0.285 \pm 0.054$	n.d. h	n.d. h	n.d. h	$0.414 \pm 0.006$	n.d. h	$1.858 \pm 0.044$	$0.179 \pm 0.001$
	-10.40%	0%	0%	0%	-15.10%	0%	-67.80%	-6.50%
WRA2 d	$0.188 \pm 0.005$	n.d. h	n.d. h	n.d. h	$0.493 \pm 0.016$	n.d. h	$1.077 \pm 0.033$	n.d. h
	-10.70%	0%	0%	0%	-28.00%	0%	-61.20%	0%
SCA e	n.d. h	n.d. h	n.d. h	n.d. h	$0.141 \pm 0.007$	n.d. h	$0.443 \pm 0.029$	n.d. h
	0%	0%	0%	0%	-24.10%	0%	-75.90%	0%
SCA2 f	n.d. h	n.d. h	n.d. h	n.d. h	$0.256 \pm 0.011$	n.d. h	$0.320 \pm 0.013$	$0.052 \pm 0.015$
	0%	0%	0%	0%	-40.70%	0%	-51.00%	-8.30%
<b>Raw materials</b>								
Brown rice koji g	$0.238 \pm 0.034$	n.d. h	$0.033 \pm 0.030$	n.d. h	$0.409 \pm 0.056$	n.d. h	$1.172 \pm 0.134$	$0.062 \pm 0.003$
	-12.40%	0%	-1.70%	0%	-21.40%	0%	-61.20%	-3.20%
White rice koji g	$0.070 \pm 0.004$	n.d. h	n.d. h	n.d. h	$0.287 \pm 0.027$	n.d. h	$1.017 \pm 0.054$	$0.068 \pm 0.006$
	-4.90%	0%	0%	0%	-19.90%	0%	-70.50%	-4.70%
Brown powder rice	$11.69 \pm 0.360$	$0.528 \pm 0.022$	$2.302 \pm 0.044$	n.d. h	$7.180 \pm 0.142$	$0.407 \pm 0.008$	$10.97 \pm 0.151$	$0.385 \pm 0.016$
	-34.90%	-1.50%	-6.90%	0%	-21.50%	-1.20%	-32.80%	-1.20%



Gelatinized brown rice powder	2.070 ± 0.459	0.101 ± 0.018	0.439 ± 0.076	n.d. h	1.115 ± 0.198	n.d. h	2.053 ± 0.401	n.d. h
	-35.80%	-1.70%	-7.60%	0%	-19.30%	0%	-35.50%	0%

**Table 1:** Vitamin E composition in amazake and its raw materials (µg/g dry food). a: All data were presented as mean ± S.D. (n=3), b: provided by Yamato Soy Sauce & Miso, c: provided by Ayumasamune Co., d: provided by Fukumitsuya Co., e: provided by Morinaga Co., f: provided by Melodian Co., g: Incubate at 57°C for 24 h., h: not detected. Values in parentheses mean the percent distribution of each vitamin E homologues.

#### 4-VG and FA levels, and DPPH radical scavenging capacity, of amazake and raw material extracts

The DPPH radical scavenging activities of hydrophobic extracts from each food sample were investigated (Table 2). The DPPH radical scavenging activity decreased in the order brown rice powder, BRA, WRA, and brown rice koji, which correlated with the order of total vitamin E contents. As the reaction ratio of α-tocopherol and DPPH radicals was 1:2 [24], the DPPH amount scavenged by vitamin E in BRA extract was calculated as approx. 78 nmol/g. This estimation was based on the assumption that the reaction ratio of each vitamin E molecular species was identical. In contrast, the amount of DPPH radical scavengers in BRA extract was about 24 times higher than the calculated value, which suggested that BRA extract should contain additional hydrophobic antioxidants to vitamin E compounds. The peak intensity of 4-VG in BRA extract was comparatively high, such that 4-VG was thought to be a possible candidate for the additional antioxidant. The amount of 4-VG in BRA was determined by NP-HPLC (2.635 ± 0.157 nmol/g). This amount was too low to explain the contribution of 4-VG to the DPPH radical scavenging capacity in BRA. The amount of FA in BRA was determined because FA was thought to be a synthetic precursor of 4-VG through a decarboxylase reaction. BRA was treated with alkaline water to release FA, and free FA was

quantified by RP-HPLC. The amount of FA in BRA was 1217 ± 30 nmol/g, which was approximately 30 times higher than that of total vitamin E, and approximately 64% of the antioxidant capacity in BRA. BRA extracts contained multiple molecular species of vitamin E and polyphenols, such as FA and 4-VG, which functioned as DPPH radical scavengers with different modes. In contrast, the DPPH radical scavenging capacity in WRA extract was very low, at only 16% of that of the BRA extract. This was due to the low amounts of vitamin E and FA in WRA. The DPPH radical scavenging capacity of SCA was the smallest of all, which was consistent with its very low vitamin E and 4-VG contents and no FA content. The DPPH radical scavenging activity of brown rice koji extract was similar to that of the WRA extract, which was consistent with the total vitamin E and FA contents in these food extracts. The trend of DPPH radical scavenging capacity roughly correlated with FA content, which was consistent with our previous report [16]. The amount of FA in brown rice powder was higher than that in brown rice koji, while the amount of 4-VG in brown rice powder was much lower than that in brown rice koji, suggesting the biotransformation of FA to 4-VG during koji and/or BRA production. The total amount of FA and 4-VG in koji was much smaller than expected, which indicated the further metabolism of 4-VG to vanillin [25] and/or loss via vaporization during the drying process.

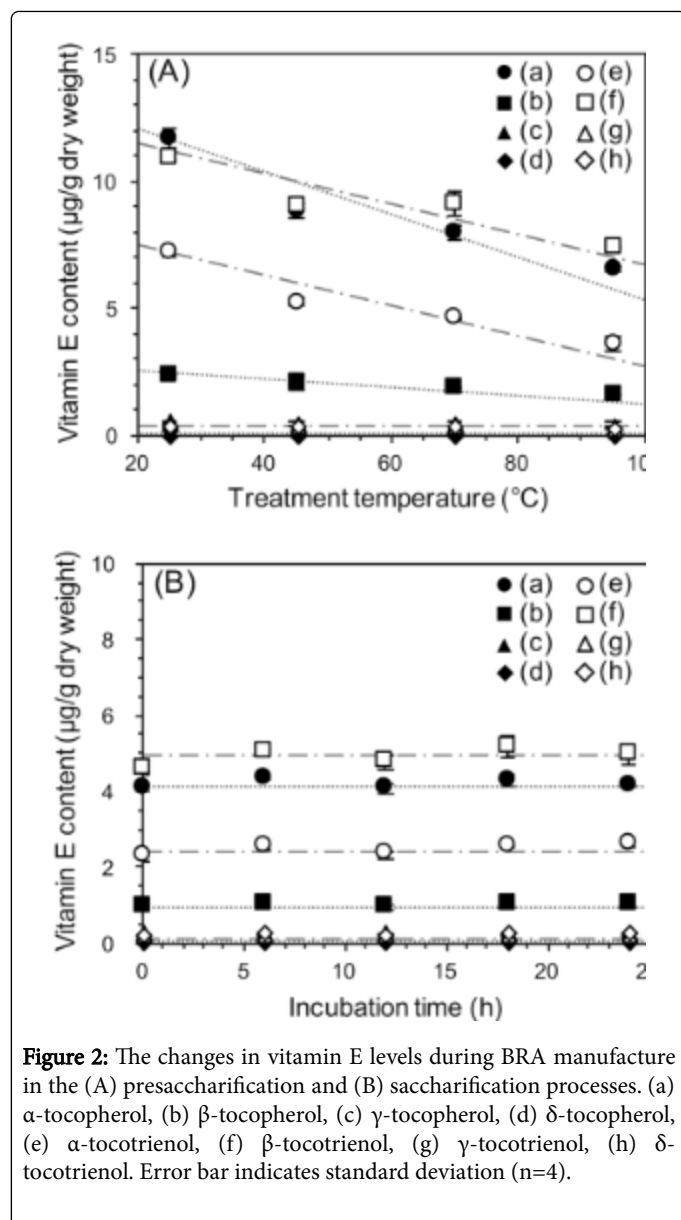
Food sample	Total vitamin E	FA	4-VG	DPPH radical
				Scavenging capacity
BRA	39.29 ± 0.762	1217 ± 30	2.635 ± 0.157	1900
WRA	6.612 ± 0.219	370.8 ± 11.6	0.129 ± 0.016	302
SCA	1.411 ± 0.086	n.d. c	2.480 ± 0.053	285
Brown rice powder	79.50 ± 1.41	1982 ± 76	0.033 ± 0.058	8660
Brown rice koji b	4.607 ± 0.587	362.2 ± 9.9	5.762 ± 0.305	325

**Table 2:** Content of liposoluble antioxidants and DPPH radical scavenging capacity a (nmol/g dry food).

#### Vitamin E levels at each step of BRA production

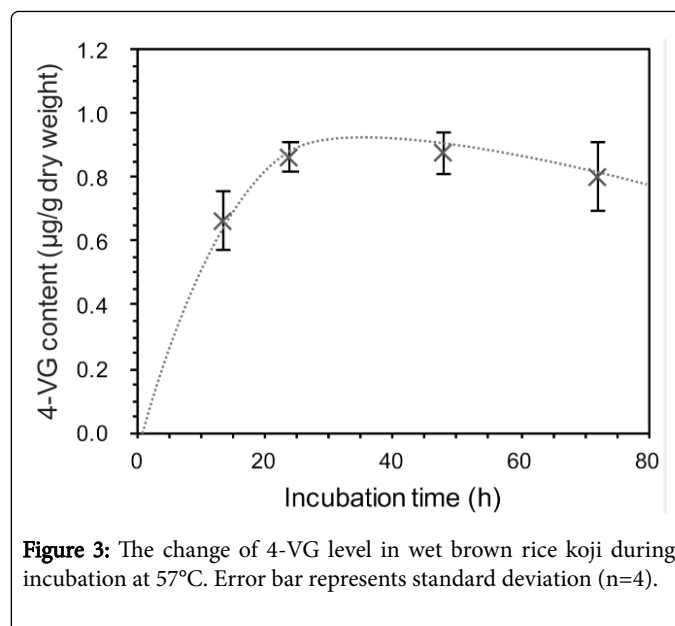
To investigate the changes in vitamin E during BRA production, HPLC analysis was performed during the processing steps, namely the presaccharification step, which included enzyme treatment and starch gelatinization (Figure S5), and the saccharification step using koji (Figure S6). Figure 2 summarizes the changes in vitamin E levels during BRA manufacture in the (A) presaccharification and (B) saccharification processes. In the presaccharification step, brown rice powder was treated with an enzyme mix containing amylase and cellulase to decrease the sample viscosity. During the presaccharification step, tocopherol and tocotrienol were decreased by the enzyme treatment at 45 and 70°C for 1 h, respectively. After

enzyme treatment, the sample was heated at 95°C for 1 h to inactivate the enzymes and gelatinize the starch. The vitamin E content also decreased during heat treatment. In the next step (saccharification), dry brown rice koji and water were directly added to the paste of gelatinized brown rice powder and incubated for over 24 h. The Brix value was monitored to verify successful saccharification by brown rice koji. After 6 h of saccharification, the level of sucrose had increased significantly, and gradually increased thereafter over 24 h (Figure S7). The vitamin E contents in the glycolated solution were unchanged during saccharification for 24 h (Figure 2B).

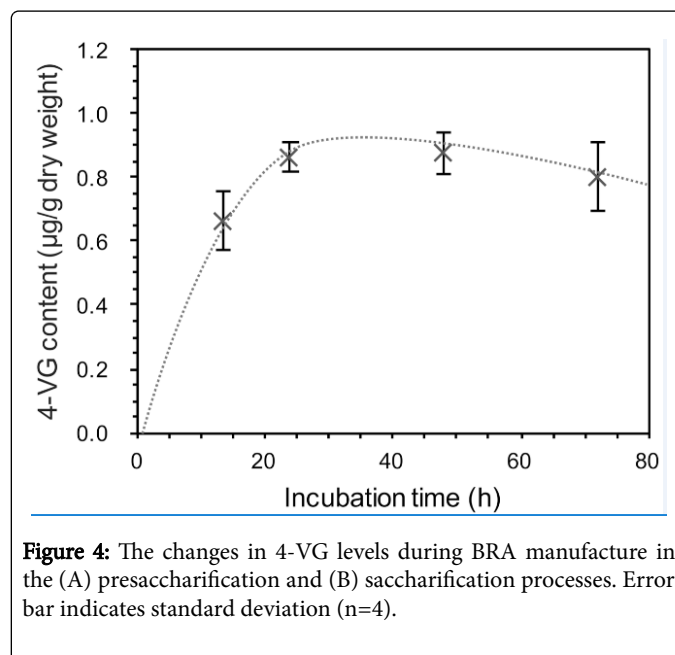


#### 4-VG levels during BRA production

4-VG was detected as a fluorescent substance in BRA and brown rice koji extract, but not detected in brown rice powder (Figure 1). To test the production of 4-VG by koji mold, the brown rice koji was incubated at 57°C and the 4-VG amount was determined by NP-HPLC. The amount of 4-VG increased during incubation, reaching the maximum level at 24 h and gradually decreased thereafter (Figure 3). *Aspergillus* sp. releases FA bound to xylan by the action of ferulic acid esterase [26] and then produces 4-VG by the action of ferulic acid decarboxylase [27]. The  $\gamma$ -oryzanol content, which is a typical polyphenol in brown rice, is known to be approx. 10–37 times higher than vitamin E contents [28]. Therefore, it can be assumed that FA esters such as  $\gamma$ -oryzanol are metabolized to produce 4-VG by the biological activities of *A. oryzae* during brown rice koji and BRA processing.



The amount of 4-VG was measured during BRA production to verify 4-VG formation. In the presaccharification process, 4-VG was not detected when brown rice powder was treated with multi-enzyme complex at 45 °C and 70 °C (Figure 4). In contrast, it was found that 4-VG was detected in the gelatinization process of starch at 95 °C, which suggested the thermal decarboxylation of FA to form 4-VG [29]. In the saccharification process after adding brown rice koji, the 4-VG amount gradually increased as incubation proceeded (Figure 4B). These results suggest that 4-VG, a typical phenolic antioxidant, was produced from FA during the BRA manufacture process.



#### Conclusion

The vitamin E composition in amazake was determined by NP-HPLC analysis with BRA found to contain six kinds of vitamin E

molecular species, except for  $\delta$ -tocopherol and  $\beta$ -tocotrienol, while four kinds of vitamin E molecular species were detected in WRA. The total level of vitamin E in BRA was six-fold higher than that in WRA, and vitamin E species in BRA were derived from brown rice powder in its raw materials. The DPPH radical scavenging activity of BRA extract was greater than that of WRA, which was consistent with the total vitamin E and FA contents. 4-VG was detected in amazake and koji samples, and can be produced from FA via enzymatic decarboxylation. The amount of 4-VG suggested that its contribution to the radical scavenging capacity was low, and FA was an abundant phenylpropanoid with radical scavenging activity in brown rice. The vitamin E levels gradually decreased during presaccharification, but were not changed during saccharification in the BRA production process. Food processing of brown rice via saccharification is thought to be a superior method for retaining antioxidative compounds. This traditional fermentation process using koji may be applicable to other vitamin E-rich grains to develop unique and novel functional foods.

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