

Changes in plasma concentration of flavonoids after ingestion of a flavonoid-rich meal prepared with basic foodstuffs

Ryo Mannen¹, Michiko T. Yasuda², Ayami Sano³, Toshinao Goda¹, Kayoko Shimoi¹, Yoko Ichikawa^{1*}

¹Graduate School of Integrated Pharmaceutical and Nutritional Sciences, University of Shizuoka, Yada 52-1, Suruga-ku, Shizuoka 422-8526, Japan; ²Department of Human Nutrition, School of Life Studies, Sugiyama Jogakuen University, 17-3 Hoshigaoka Motomachi, Chikusa-ku, Nagoya 464-8662, Japan; ³Department of Health and Nutritional Sciences, Faculty of Health Promotional Sciences, Tokoha University, 1230 miyakodacho Kita-ku, Hamamatsu, Shizuoka, 431-2102, Japan

Corresponding author: Yoko Ichikawa, PhD, Graduate School of Integrated Pharmaceutical and Nutritional Sciences, University of Shizuoka, Yada 52-1, Suruga-ku, Shizuoka 422-8526, Japan

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ABSTRACT

Background: As flavonoids have a variety of functions, such as antioxidant activity, there is a growing interest in the development of flavonoid supplements. However, there have been reports of DNA damage due to exposure to flavonoids at high concentrations in rats, which could suggest that a habitual intake of flavonoid supplements may cause toxicity. Therefore, we considered that ingesting flavonoids from a typical meal combined with basic foodstuffs is safe because it is unlikely to result in high concentrations like supplements and focused on the intake of flavonoids from a typical meal. Thus, this study investigated the absorption of flavonoids in humans after the consumption of a typical meal.

Methods: On the first 2 days of the study, seven healthy volunteers were provided with meals low in flavonoids (flavonoid content below the detection limit by HPLC: less than 0.24 mg/meal) three times a day as a washout. A flavonoid-rich meal (40.44 ± 1.49 mg/meal) was then provided for breakfast on the third day. Blood was collected from all volunteers 0, 2, 3, 7, 8, and 9 h after the flavonoid-rich meal was consumed. After enzyme hydrolysis of the plasma, the plasma concentrations of flavonoids aglycone of quercetin, daidzein and genistein were measured using LC-MS. Urine was also collected and pooled 24 h after the flavonoid-rich meal was consumed. Thereafter, the urine was treated with enzyme hydrolysis, and the measurement of urinary flavonoids was performed.

Results: Plasma flavonoid peaks were observed 8 h after consumption of the flavonoid-rich meal (quercetin: 4.29 ± 1.46 μ M, daidzein: 0.51 ± 0.41 μ M, genistein: 0.91 ± 0.73 μ M). Furthermore, flavonoids were confirmed to be present in plasma even at 9 h after the intake meal. The urinary recovery of flavonoids was $3.43 \pm 1.50\%$ for quercetin, $13.87 \pm 6.68\%$ for daidzein, and $16.89 \pm 11.40\%$ for genistein.

Conclusion: These results suggest that consuming a typical meal that combines a variety of basic foodstuffs delays attainment of the plasma flavonoid peak compared with consuming a single type of food or supplements as previously reported. In addition, the flavonoid urinary recovery was also reduced compared with those previously reported.

Trial Registration: UMIN000037001

Key words: Flavonoids, plasma concentration, flavonoid-rich meal, human study

BACKGROUND

Flavonoids are a type of polyphenols and are categorized into the following classes based on their aglycone structure: flavonols, isoflavones, flavones, flavanols, anthocyanins, and flavanones. Flavonoids exist as glycosides in plants and approximately 9000 types of flavonoid glucosides have been reported to date [1]. Flavonoids exhibit various functions such as antioxidant activity and apoptosis induction [2, 3]. As they are found in a lot of vegetables and fruits, it is thought to be easy to consume them regularly. In addition, dietary flavonoids have potentially beneficial effects in preventing various diseases such as heart disease, cancer, and obesity [4-6]. In their study, Vinson et al. reported how quercetin, a flavonoid found in high concentrations in onions, inhibits

the oxidation of low-density lipoprotein (LDL) [7]; this effect is expected to reduce the factors of lifestyle-related diseases. In their study on a cohort of Dutchmen, Hertog et al. reported that the risk of coronary diseases was lower in the group with a high flavonoid intake than that in the group with a low flavonoid intake [8]. Thus, the effect of the bioactivities of flavonoids on various diseases is expected by many people, and there have been developments in the market of food with health-promoting benefits for flavonoids.

However, there are concerns about the promotive effect for oxidation of DNA by excessive intake of antioxidants. For example, it has also been reported that exposure to high concentrations of quercetin can induce DNA damage in rats [9]. Furthermore, it has been reported in vitro that daidzein and genistein metabolites damage DNA [10]. In addition, it has also been reported that an excessive intake of quercetin in rats and pigs causes accumulation in various organs [11], indicating that the excessive intake of antioxidants including flavonoids could be problematic. Although supplements may be an effective way of ingesting flavonoids, caution against overdosing should be exercised. On the other hand, it would be safer to obtain flavonoids from a meal comprising several basic foodstuffs because the probability of the concentration of flavonoids in such a meal being as high as that in a supplement is very low.

The Academy of Nutrition and Dietetics recommends that a functional ingredient should be ingested by combining various basic foodstuffs rather than ingesting a single type of food, nutrient, or supplement [12] (Total Diet Approach). In addition, Azuma et al. [13] fed stress-burdened rats with either quercetin supplements or onions that possessed an equal amount of quercetin aglycone to that of the supplement; they observed that oxidative conditions, shown by thiobarbituric acid reactive substance which is an oxidative marker, were lower in the group fed with onions than in the group fed with the quercetin supplement. This result indicates that consuming flavonoid-containing food may have an anti-oxidative effect, an effect not indicated in consuming only the functional ingredient flavonoid in supplement form. Based on these reports, it is considered important to examine the efficacy of intaking flavonoids from a meal rather than a supplement. Furthermore, in order to use flavonoids intake from the typical meal for human health effects, it is necessary to reveal the changes in the absorption and blood concentration of flavonoids following flavonoid intake from a typical meal.

Recent studies have provided further clarity of the mechanisms of the absorption and metabolism of flavonoids. Quercetin is converted into aglycone and absorbed in the small intestine by lactase phlorizin hydrolase (LPH) and a sodium-dependent glucose transporter-1 (SGLT-1) mediated-transport system [14]. Thereafter, it is metabolized to conjugate in the small intestinal

mucosal epithelium and is then transported to the liver and metabolized into methylated forms [15]. In contrast, quercetin glycosides that are not absorbed in the small intestine reach the large intestine. Most of them are excreted in feces, but some are converted to aglycones by intestinal bacteria and absorbed [16]. Isoflavones such as daidzein and genistein are also reported to be absorbed in the small or large intestines similar to quercetin. Isoflavones are absorbed after cleavage of sugar chains by intestinal bacteria and enzymes. Thereafter, they are converted to glucuronic acid or sulfate conjugates and then converted to metabolites during circulation in the body [17, 18]. Although there have been various reports on the absorption and metabolism mechanisms of flavonoids, these have been carried out using only flavonoid supplements or single type of food in cells, animals, and humans [19]. However, humans usually consume flavonoids from meals comprising a variety of basic foodstuffs and which are cooked in various ways, such as boiling, grilling, frying, and steaming. These meals also comprise ingredients such as fats and dietary fibers, which may influence the internal change of flavonoids. Therefore, it is thought that the absorption rate differs among the intake of flavonoids from a typical meal, supplements, or single type of food. Thus, in cases where internal changes in flavonoids are being assessed using actual eating habits, other nutrients also need to be taken into consideration. However, to the best of our knowledge, we know of few reports that have examined flavonoids consumption from typical meals rather than single ingredients or a single type of food.

In this study, we focused on dietary flavonoids such as quercetin, kaempferol, daidzein, and genistein and examined the urinary excretion and plasma concentration of these flavonoids after the consumption of a typical flavonoid-rich meal, which comprised a staple food, main dish, side dish, and soup, and was designed based on the Dietary Reference Intakes (DRIs) for Japanese (2015) [20] reference values for energy and those for the ratio of nutrients to energy availability.

METHODS

Test meals

Hertog et al. [8] and Knekt et al. [21] reported that flavonoid intakes were 25.9 mg/day and 3.4 mg/day for Dutch and Finnish, respectively. Arai et al. [22] reported a flavonoid intake of 63.9 mg/day (flavonols and flavones: 16.7 mg/day, isoflavones: 47.2 mg/day) in 117 healthy Japanese people. Therefore, it is estimated that the flavonoid intake in this Japanese population was 21.3 mg/meal (flavonols and flavones: 5.6 mg/meal; isoflavones: 15.7 mg/meal). The flavonoid-rich meal in this study was prepared with basic foodstuffs referred to the flavonoids content of each foodstuff reported and prepared to contain more flavonoids than the 21.3 mg/meal reported above.

The flavonoid-rich meal was formulated according to the menu shown in Table 1. Three factors were considered in formulating the meals: (1) they should satisfy nutrition requirements based on the DRIs for Japanese (2015) [20]; (2) they should comprise flavonoid-rich basic foodstuffs; and (3) they should be modeled after typical Japanese meals, which comprise a staple food, main dish, side dish, and soup. The nutritional value of the meal was calculated using the Standard Tables of Food Composition in Japan, seventh edition (2015) [23] (data not shown).

The low-flavonoid meals that were ingested as part of the washout procedure used in this study were similar to those used in the study by Sakakibara et al. [24]. The flavonoid content of the low-flavonoid meals was below the detection limit by high performance liquid chromatography (HPLC).

Table 1. Dishes comprising the flavonoid-rich meal

Menu	Ingredients (g / dish)
Rice	rice (80)
Tofu with a thick vegetable sauce	firm tofu (100), wheat flour (10), onion (20), carrot (10), shiitake mushroom (10), oil (3), dashi (60), soy sauce (6), sweet sake (3), potato starch (1)
Onion salad	onion (80), dashi (0.5), soy sauce (7.5), vinegar (5), sesame oil (4.5)
Pak-choi seasoned with wasabi and vinegar	Pak-choi (50), soy sauce (5), grain vinegar (5), dashi (5), wasabi (2)
Onion miso soup	onion (20), rice miso (8), dashi (150)
Apple	apple (100)

Chemicals

Quercetin, kaempferol, apigenin, luteolin, naringenin, daidzein, genistein, flavone, and robinetin were procured from Extrasynthese (Genay, France). In general, as flavonoids are present as individual glycosides in plants enzyme treatment to samples of meal, plasma and urine is needed to convert conjugate to aglycone. In this study β -Glucosidase (crude solution from almonds, G0395) and β -Glucuronidase (crude solution from *Helix pomatia*, type H-5, G1512) were used for this purpose. In addition, the amount of flavonoids in this study used was the total amount that converted to aglycone by treating the plasma, urine, and meal with a deconjugated enzyme treatment. β -Glucosidase (crude solution from almonds, G0395) and β -Glucuronidase (crude

solution from *Helix pomatia*, type H-5, G1512) were purchased from Sigma Aldrich (St. Louis, MO, USA). All other chemicals used in the study were of liquid chromatography-tandem mass spectrometry (LC-MS) or HPLC grade. The food materials used to prepare the meals were purchased from a local supermarket.

Study design

The study protocol was approved by the Ethics Committee of the University of Shizuoka (permission number 26-12). The subjects included seven healthy volunteers (four males and three females, aged, 22–27 years) who gave informed consent (Table 2). The participants were asked to refrain from eating or drinking anything other than the test meals and water during the study period. On the first 2 days of the experiment, all participants consumed low-flavonoid meals. On the third day, all participants consumed the flavonoid-rich meal after fasting overnight. In addition, they also consumed the low-flavonoid meals detailed in Menus 2 and 3 for lunch and dinner, respectively. Venous blood samples (5 mL) of all volunteers were collected in heparinized tubes 0, 2, 3, 7, 8, and 9 h after the flavonoid-rich meal was consumed and immediately centrifuged at $3000 \times g$ for 10 min to obtain the plasma. Urine samples were also collected at 24 h after the flavonoid-rich meal was consumed and pooled using URIN-MATEP (Sumitomo Chemical Co. Ltd., Tokyo, Japan). The total volume of the collected urine was also measured. Plasma and urine samples were stored at -80°C until use.

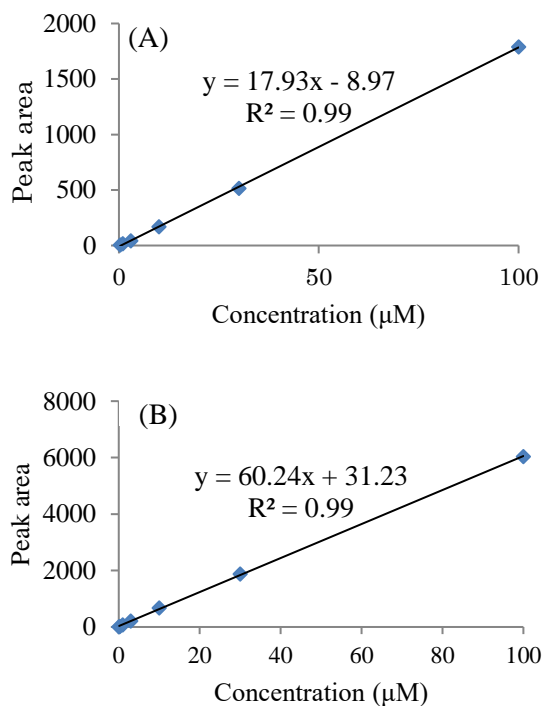
Table 2. Characteristics of participants (n = 7)

	Mean \pm SD
Age (y)	22.3 \pm 1.8
Body weight (kg)	58.7 \pm 11.0
Body Mass Index (kg/m ²)	21.3 \pm 2.2

Extraction of meals

The flavonoid-rich meal and low-flavonoid meals were analyzed to determine their flavonoid content (n = 3). Prior to the analysis, meals were frozen in liquid nitrogen and then lyophilized to a powder. These samples were subjected to extraction according to the method reported by Sakakibara et al. [25] with modifications. Briefly, 50-mg dry powder was incubated with 37 U of β -glucosidase in 2-mL citric acid–phosphoric acid buffer (pH 4.6) for 4 h at 37°C . Flavone (final

concentration, 0.05 μM) was added to the sample as an internal standard. Samples were then mixed with 6-mL methanol for 1 min using a vortex mixer. The mixture was then centrifuged at $3000 \times g$ at 4°C for 10 min. The supernatant was collected, and the pellet was re-extracted three times using 2 mL of 90% methanol in water. Methanol supernatants so obtained were combined and reduced to dryness in vacuo. This dried extract was further dissolved in 1-mL 0.5% trifluoroacetic acid (TFA) in methanol, before centrifugation at $15,000 \times g$ at 4°C for 10 min. After this the supernatant was analyzed using a UV detector (G4303A; Agilent Technologies Inc., Santa Clara, CA, USA) connected to an Agilent 1200 HPLC apparatus (Agilent Technologies Inc., Santa Clara, CA, USA) using a C18 column (particle size, 2.7 μm ; dimensions, 2.1×150 mm; Agilent Technologies Inc., Santa Clara, CA, USA). The flavonoids measured were quercetin, kaempferol, daidzein, genistein, and flavone. In addition, apigenin, luteolin, and naringenin were measured because they may be detected based on previous reports [8, 21, 22]. Detection limits for these flavonoids were 0.1 μM . A gradient program using solvent A (0.1% formic acid) and solvent B (acetonitrile) was used at a flow rate of 0.6 mL/min. The gradient program was as follows: 0–2.5 min, linear gradient from 5% to 15% B; 2.5–2.51 min, linear gradient to 20% B; 2.51–20 min, hold 20% B; 20–25 min, linear gradient to 100% B; 25–26 min, linear gradient to 5% B; and 26–29 min, hold 5% B. The measured concentrations of the flavonoids standard solution using HPLC were 0.1 μM , 0.3 μM , 1 μM , 3 μM , 10 μM , 30 μM , and 100 μM . These flavonoids standards were measured three times to create a calibration curve (Figure 1).



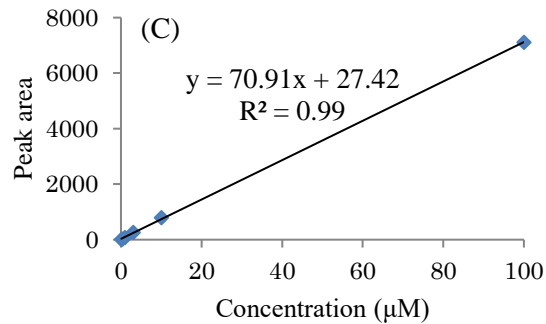


Figure 1. Calibration curve of quercetin standard (A), daidzein standard (B), and genistein standard (C) using HPLC. The measured concentrations of each flavonoid standard solution were 0.1 µM, 0.3 µM, 1 µM, 3 µM, 10 µM, 30 µM, and 100 µM. The flavonoids standards were measured three times.

Plasma extraction

Flavonoids in the plasma collected were extracted as described by Murota et al. [26] with slight modifications. Briefly, 150-µL plasma was incubated with 300 U of β-glucuronidase from *Helix pomatia* in 200-µL 0.1 M sodium acetate buffer (pH 5.0) and 30-µL 50 mM ascorbic acid for 30 min at 37°C. Robinetin (final concentration, 0.5 µM) was added to the sample as an internal standard. Samples were then mixed in 1-mL ethyl acetate for 1 min using a vortex mixer. The mixture was then centrifuged at 15000 × g at 4°C for 10 min. The supernatant obtained was collected, and the pellet was re-extracted three times using 1 mL of ethyl acetate. The ethyl acetate supernatants were combined and reduced to dryness in vacuo. The dried sample was dissolved in 200-µL 0.5% formic acid in methanol, before centrifugation at 15000 × g at 4°C for 10 min. Next, the sample was analyzed for quercetin, kaempferol, apigenin, luteolin, naringenin, daidzein, genistein, and robinetin using a Q Exactive LC-MS (Thermo Fisher Scientific, Waltham, MA, USA). The mass spectrometer was operated in the negative-ion electrospray ionization (ESI) mode with targeted selected-ion monitoring (SIM) mode for all samples. The aforementioned flavonoids were determined using an ultra performance liquid chromatography (UPLC) (Accela; Thermo Fisher Scientific) with an ACQUITY UPLC BEH C18 column (particle size, 1.7 µm; dimensions, 2.1 × 100 mm; Waters, MA, USA). The detection limits for these flavonoids were all 0.1 µM. A gradient program using solvent A (0.1% formic acid) and solvent B (acetonitrile) was used at a flow rate of 0.4 mL/min. The gradient program was as follows: 0–1 min, hold 2% B; 1–2 min, linear gradient to 23% B; 2–12 min, hold 23% B; 12–18 min, linear gradient to 100% B; 18–21 min, hold 100% B; 21–22 min, linear gradient to 2% B; and 22–25 min, hold 2% B. The measured concentration of the flavonoids standard solution using LC-MS were 0.1 µM, 1 µM, and 10 µM. These flavonoids standards were measured three times to create a calibration curve (Figure 2).

Extraction of urine

Flavonoids in the urine collected were extracted in accordance with a method reported by Kenneth et al. [27] with modifications. Briefly, 5 mL of urine was incubated with 100 U of β -glucuronidase from *Helix pomatia* in 500 μ L of 0.1 M sodium acetate buffer (pH 5.0) and 1 mL of 50 mM ascorbic acid at 37°C for 30 min. Flavone (final concentration, 0.5 μ M) was added to the sample as an internal standard. The solution was applied to a Sep-Pak C18 cartridge (Waters, Milford, MA, USA), which had been pretreated by passage with methanol. The cartridge was washed with 0.01 M oxalic acid and distilled water, and the methanol eluate was obtained. The methanol eluate was then evaporated to dryness. The dried sample was dissolved in 200 μ L of 0.5% formic acid in methanol, followed by centrifugation at 15000 \times g at 4°C for 10 min. The supernatant was analyzed using LC-MS as previously discussed.

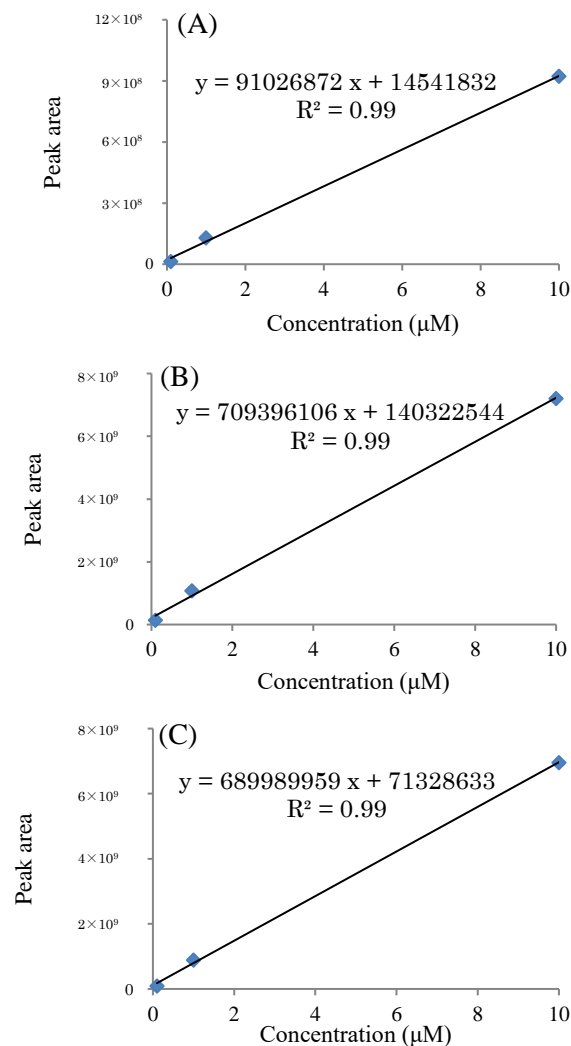


Figure 2. Calibration curve of quercetin standard (A), daidzein standard (B), and genistein standard (C) using LC-MS. The measured concentrations of each flavonoids standard solution were 0.1 μ M, 1 μ M and 10 μ M. The flavonoids standards were measured three times.

Statistical analysis

Data were analyzed using the Statistical Analysis System software (SPSS, version 22.0, Chicago, IL). All the values are presented as means \pm SD. Dunnett's post hoc test was used to analyze the data obtained. Results were considered significant at $p < 0.05$.

RESULTS

Analysis of meals

The flavonoid content of the flavonoid-rich meal is shown in Table 3. The total flavonoid content calculated from the combined value of the content of each of the flavonoids detected was 40.44 ± 1.49 mg/meal. This flavonoid content was approximately 2 times higher than the daily flavonoid intake of 21.3 mg/meal in a Japanese population in a previous study [22]. Among all flavonoids investigated in this study, genistein was the highest (14.26 ± 1.05 mg/meal), followed by daidzein (11.79 ± 0.33 mg/meal), quercetin (11.72 ± 0.13 mg/meal), and kaempferol (2.67 ± 0.10 mg/meal). The other flavonoids (apigenin, luteolin, naringenin) were below the detection limit in these experimental conditions.

Table 3. Flavonoid content of the flavonoid-rich meal.

Menu	Quantity of flavonoids (mg / meal)				
	Quercetin	Kaempferol	Daidzein	Genistein	Total
Rice	ud	ud	ud	ud	0.00
Tofu with a thick vegetable sauce	1.85 ± 0.04	ud	7.00 ± 0.10	9.36 ± 0.10	18.21 ± 0.17
Onion salad	6.22 ± 0.01	ud	ud	ud	6.22 ± 0.01
Pak-choi seasoned with wasabi and vinegar	ud	ud	ud	ud	0.00
Onion miso soup	3.12 ± 0.11	ud	4.79 ± 0.39	4.90 ± 1.12	12.81 ± 1.61
Apple	0.53 ± 0.01	2.67 ± 0.10	ud	ud	3.2 ± 0.01
Total	11.72 ± 0.13	2.67 ± 0.10	11.79 ± 0.33	14.26 ± 1.05	40.44 ± 1.49

ud: under the detection limit

Apigenin, luteolin, and naringenin were below the detection limit in these experimental conditions.

Flavonoid concentration in plasma

Quercetin was detected in all plasma samples, except those obtained during fasting as shown in Figure 3A, and the highest plasma concentration of quercetin, $4.29 \pm 1.46 \mu\text{M}$, was observed at 8 h after consumption of the flavonoid-rich meal (Table 1). Significant differences were noted between the concentrations obtained at 0 and 2 h, 0 and 3 h, 0 and 7 h, 0 and 8 h, 0 and 9 h after meals were consumed. Daidzein (Figure 3B) and genistein (Figure 3C) were detected in all plasma samples. Significant differences were noted between the concentrations obtained at 0 and 8 h, 0 and 7 h after meals were consumed, respectively. In addition, the highest plasma concentrations of daidzein and genistein ($0.51 \pm 0.41 \mu\text{M}$ and $0.72 \pm 0.39 \mu\text{M}$, respectively) were observed at 8 h after meals. Apigenin, luteolin, and naringenin, which were not detected in the flavonoid-rich meal, were not detected in plasma samples.

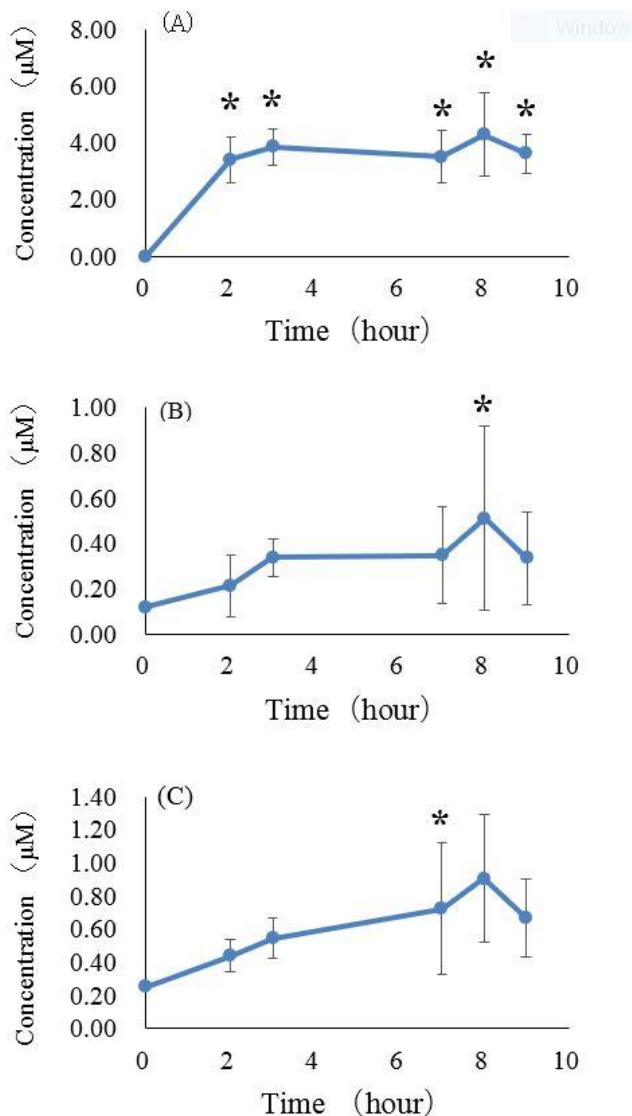


Figure 3. Concentrations of quercetin (A), daidzein (B), and genistein (C) in plasma collected

from seven human subjects 0–9 h after consumption of a flavonoid-rich meal. Values are means \pm standard deviation ($n = 7$). The asterisk indicates a significant difference vs 0 hours, $p < 0.05$.

Urinary excretion of flavonoids

In this study, we considered that flavonoids excreted in the urine were once absorbed in the body. Therefore, the urinary recovery was calculated as the ratio of urinary excretion relative to the amount of flavonoids in the flavonoid-rich meal. The average urinary recovery of quercetin was $3.43 \pm 1.50\%$ (range, 0.26%–4.52%) (Table 4). The average urinary recovery of daidzein and genistein, $13.87 \pm 6.68\%$ (range, 5.60%–25.36%) and $16.89 \pm 11.40\%$ (range, 5.19%–38.85%), respectively, were higher than that of quercetin. Individual differences in isoflavones (Daidzein and Genistein) were also large. Apigenin, luteolin, and naringenin, which were not detected in the flavonoid-rich meal, were not detected in urine samples (data not shown).

Table 4. Urinary flavonoid excretion amounts and recovery rates at 24 h after flavonoid-rich meal consumption

	Quercetin	Daidzein	Genistein
Intake (total, mg)	11.72 ± 0.13	11.79 ± 0.33	14.26 ± 1.05
Urinary excretion (mg)	0.24 ± 0.18	2.41 ± 1.78	1.68 ± 0.87
Urinary recovery (%)	3.43 ± 1.50	13.87 ± 6.68	16.89 ± 11.40

Values are presented as the means \pm SD.

DISCUSSION

In this study, the intake source of flavonoids was a typical meal comprising basic foodstuffs used in Japan. Generally, typical meals in Japan are prepared using various ingredients and different cooking operations such as boiling, baking, and frying. Therefore, they can contain various nutrients and components that can influence the absorption of flavonoids. In particular, nutrients such as lipids and dietary fibers have been reported to affect the absorption of flavonoids. The presence of lipids delays the transfer of stomach contents within the intestinal lumen [28, 29]. However, it was reported that the quercetin urinary recovery was improved by the simultaneous intake of lipids, because the presence of lipids also increases the absorption by increasing solubility in lipid micelles [29]. Dietary fiber has generally been shown to delay absorption as it inhibits the diffusion of food components in the lumen. In addition, dietary fiber inhibits absorption of

flavonoids itself [30]. The DRIs [20] for Japanese (2015) recommends the following amounts of these nutrients be included in each meal: lipids, 15–25g/meal; and dietary fiber, 6–7g/meal. Because of this, meals can contain more components that may affect the absorption of flavonoids compared with supplements or single type of food. In this study, 13.5g of lipids and 6.2g of dietary fiber were included in the flavonoid-rich meal as estimated from the Food Composition in Japan, Seventh Edition [23]. As a result, it is considered that the presence of quercetin, daidzein, and genistein was confirmed in the plasma even at 9 h after ingestion of the flavonoid-rich meal, and plasma concentrations of quercetin, daidzein, and genistein were highest at 8 h after ingestion of the meal (Figure 3). Manach et al. [19] conducted a meta-analysis on the bioavailability of flavonoids and showed that a plasma concentration peak was present at 0.68–1.9 h after consumption of quercetin from onions in a German study population. Additionally, Chang et al. reported a plasma concentration peak at 3.71 h for daidzein and 4.86 h for genistein following the ingestion of isoflavones from soymilk in a Korean female study population [31]. From the above, the results indicate that there was a delay of >6 h for quercetin, 4 h for daidzein, ≥ 3 h for genistein when observing the plasma flavonoid peak after consuming the typical meal compared with previous reports. In addition, Nakamura et al. [28] revealed that the plasma flavonoid peak was delayed when onion and tofu were consumed at the same time compared with being ingested separately. It is inferred that flavonoid absorption was delayed in this case because dietary fiber and lipid contents increased due to the ingestion of onion and tofu at the same time. In this study, the test meal was conducted using many basic foodstuffs and seasonings, therefore, it was a typical meal containing a large amount of lipids and dietary fiber, which may have affected the absorption of flavonoids, compared with the test foods used in the studies by Manach et al. [19], Chang et al. [31], and Nakamura et al. [28]. From the results it was inferred that the quercetin, daidzein, and genistein peaks in the plasma were delayed than when single food ingredients were ingested.

The urinary recovery of flavonoids in the body were 0.26%–4.52% for quercetin, 5.60%–25.36% for daidzein, and 5.19%–38.85% for genistein in the urine samples (Table 4). In contrast, Manach et al. [19] reported a quercetin urinary recovery of 0.07%–6.4% in their meta-analysis. In addition, Chang et al. [31] reported daidzein and genistein urinary recovery of 33.8%–45.8% and 16.9%–23.4%, respectively. The urinary recovery of the flavonoids in the typical meal for this study was slightly lower compared with these reports (Table 4). Our results could suggest that flavonoid absorption may be lower following the intake of a typical meal compared with a single type of food. We considered that this decrease in urinary recovery was due to the function of the dietary fiber contained in the typical meal. As mentioned above, dietary fiber has generally been

shown to delay absorption of food components due to its inhibition of the diffusion of food components in the lumen, and in addition, dietary fiber inhibits absorption of flavonoids [30]. Therefore, it was considered that the urinary recovery of flavonoids was reduced in this study as the typical meal contained more dietary fiber than a single type of food. In contrast, it has been shown that a simultaneous intake of lipids can improve the quercetin urinary recovery [29]. However, the urinary recovery was low in this study. It is considered that this may be due to the absorption inhibitory effect of dietary fiber rather than the absorption improvement effect of lipids.

In human blood, quercetin rarely exists as aglycone and exists instead as metabolite or conjugate. At present, more than 20 quercetin metabolites in human plasma have been found [32]. Similar metabolites have also been detected in urine [33, 34]. It has been clarified from previous reports [19, 35] that there are differences in urinary recovery among quercetin metabolites and conjugates. However, flavonoids in plasma and urine in this study were treated with enzymes, and the resulting aglycone was measured as the total amount of metabolites and conjugates. Therefore, it was not possible to study the differences in the urinary recovery of metabolites and conjugates. It is necessary to measure these metabolites and conjugates to examine the differences in the detailed urinary recovery of flavonoids.

Agnes et al. [36] reported that following the administration of four doses of 500 mg of quercetin to sarcoidosis patients, a plasma quercetin concentration of 0.27 μM on average was observed the following day, indicating that plasma malondialdehyde was significantly reduced. In addition, Sarah et al. [37] reported that after 150 mg/day of quercetin capsules were administered daily for 2 weeks to healthy subjects, the plasma quercetin concentration was 0.4 μM on average, and the values of Tumor Necrosis Factor (TNF- α), uric acid, and oxidized LDL did not change. From these reports, the anti-oxidative and anti-inflammatory effects of quercetin may not be effective if inflammation is not already present as in healthy subjects, even if the concentration of quercetin is high. In this study the plasma concentration of quercetin was $4.29 \pm 1.46 \mu\text{M}$ at the highest value, which is high compared with previous reports [36, 37]. Therefore, it was suggested that humans who experience regular inflammation, such as sarcoidosis patients, may obtain an anti-inflammatory effect from consuming regular flavonoid-rich meals. However, in these reports [36, 37] the plasma was collected a full 24 h after intake of quercetin. Therefore, the plasma concentration of quercetin may not have been at its highest value unlike in this study.

In this study, individual differences were identified in the plasma concentrations and urinary recovery of the flavonoids detected. A part of flavonoids is converted to aglycones by intestinal bacteria and absorbed [16]. In addition, it has been reported that there are inter-individual

differences in intestinal bacteria and also variation within individuals [38]. Therefore, we considered that differences in intestinal bacteria might have been an influence on the absorption speed and urinary recovery of flavonoids in this study. In addition, the small number of subjects (n=7) in this study might increase individual differences. In the future, we would like to increase the number of subjects in the study and collect and consider more detailed data.

CONCLUSION

Humans usually consume flavonoids from typical meals comprising a variety of ingredients and those that are cooked in various ways, such as boiling, grilling, frying, and steaming. Therefore, such meals contain various nutrients and components that influence the absorption of flavonoids. Consuming flavonoids from such a flavonoid-rich typical meal resulted in a delayed plasma flavonoid peak compared with that observed after consuming flavonoid supplements or single type of food. The plasma flavonoid peak was noted at 8 h after the flavonoid-rich typical meal was consumed, and the presence of plasma flavonoids was confirmed even after 9 h. In contrast, the urinary recovery of flavonoids was lower than that seen following the intake of supplements or single type of food. These phenomena were considered to have been influenced by the inhibition of the diffusion and absorption of food components due to the dietary fiber present in the meal and the delay of the transfer of stomach contents due to lipids. Therefore, it was suggested that the absorption of flavonoids was delayed when they were consumed as part of a typical meal, prolonging their existence in the plasma for a longer time, and reducing their urinary recovery.

List of abbreviations Used: LDL, low-density lipoprotein; DRIs, dietary reference intakes; LC-MS, liquid chromatography-tandem mass spectrometry; HPLC, high performance liquid chromatography; TFA, trifluoroacetic acid; ESI, electrospray ionization; SIM, selected-ion monitoring; UPLC, ultra performance liquid chromatography

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