

Difference in Plasma Metabolite Concentration after Ingestion of Lemon Flavonoids and Their Aglycones in Humans

Yoshiaki MIYAKE¹, Chika SAKURAI², Mika USUDA², Syuichi FUKUMOTO³, Masanori HIRAMITSU³, Kazuhiro SAKAIDA³, Toshihiko OSAWA⁴ and Kazuo KONDO²

¹Faculty of Human Wellness, Tokaigakuen University, Nagoya 468–8514, Japan

²Institute of Environmental Science for Human Life, Ochanomizu University, Tokyo 112–8610, Japan

³Pokka Corporation Ltd., Nishikasugai-gun, Aichi 481–8515, Japan

⁴Laboratory of Food and Biodynamics Graduate School of Bioagricultural Sciences, Nagoya University, Nagoya 464–8601, Japan

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Summary The concentrations of metabolites in human plasma after ingestion of flavanone glycosides (FG) and their aglycones (FA) in lemon were examined. FG consisting abundantly of eriocitrin were prepared from lemon peel and FA consisting abundantly of eriodictyol were prepared from FG by treatment with β -glucosidase. Eriodictyol, homoeriodictyol, and hesperetin in plasma up to 4 h after ingestion of FG with water or FA with water by subjects were not detected in plasma of non-enzyme treatment but in plasma after treatment with β -glucuronidase and sulfatase. Metabolites in plasma after ingestion of FG and FA in humans were shown to exist as the glucuro- and/or sulfo-conjugates of eriodictyol, homoeriodictyol, and hesperetin. After ingestion of FA, the concentration of metabolites in plasma exhibited a high maximum peak at 1 h. The AUC (area under the blood concentration time curve) level of metabolites of FA was higher than that of FG. FA were suggested to be absorbed faster and in higher amounts than FG. The AUC of metabolites in subject plasma after ingestion of FG with flavonoid-depleted lemon juice was shown to change to a low level in comparison with that of FG with water. The maximum concentration peak of metabolites in plasma was faster at 0.5 h than FA with water but the AUC level was similar to FA with water, when subjects ingested FA with vodka (40% ethanol). The absorption hour of FG and FA was shown to be affected by the co-existing solution.

Key Words lemon flavonoid, eriocitrin, plasma, human, ingestion

Epidemiological studies indicate a protective relationship between the consumption of citrus fruits or juice and the risk of ischemic stroke and lung cancer (1, 2). Some fundamental studies support a protective effect provided by flavonoid consumption in these diseases (3–6). Citrus fruits contain various kinds of flavonoids such as flavanone glycoside, flavone glycoside, and polymethoxyflavone (3, 4). Flavonoids in lemon fruit (*Citrus limon* BURM. f.) have been reported to be such flavanone glycosides as eriocitrin (eriodictyol 7-O- β -rutinoside) and hesperidin (hesperetin 7-O- β -rutinoside) and such flavone glycosides as diosmin (diosmetin 7-O- β -rutinoside) and 6,8 C-diglucoyldiosmetin (7–10). Eriocitrin has been reported to be the most abundant flavonoid in lemon fruit and to have the highest antioxidative activity of the flavonoids in citrus fruits in vitro (9, 11). It was also shown to have a suppressive effect for oxidative stress in the liver of streptozotocin-induced diabetic rats (12) or exercise-induced rats (13).

For the study of functional compounds such as flavonoids in foods, it is important to determine how the compounds in food are metabolized and absorbed in vivo and how the metabolites function in a living sys-

tem. There have been several human studies that have investigated the metabolism and absorption of flavonoids (14), such as anthocyanin (15, 16), flavonol (17, 18), isoflavone (19), and flavanone (20–22). As for eriocitrin, it was reported for metabolites by human intestinal bacteria (23) and for metabolites in plasma and urine after oral administration of eriocitrin in rats (24). In this study, flavanones in plasma after oral administration of flavanone glycosides (FG) consisting abundantly of eriocitrin in humans were determined by HPLC for the purpose of examination of the metabolites and absorption characteristics of eriocitrin in humans for the first time. When flavanone aglycones (FA) consisting abundantly of eriodictyol were ingested in humans, the difference in absorption characteristics after ingestion of FG or FA was also examined. Flavonoid metabolites in plasma were reported to be detected at an early hour after ingestion of flavonoids in humans (15–19). We examined the metabolites in plasma up to 4.0 h after ingestion of FG with water and FA with water in humans in this study. Furthermore, flavanones in plasma after ingestion of FG with lemon juice or FA with an alcoholic drink were determined by HPLC for the purpose of examination of absorption characteristics for co-existing solutions of FG and FA.

E-mail: miyakeyo@tokaigakuen-u.ac.jp

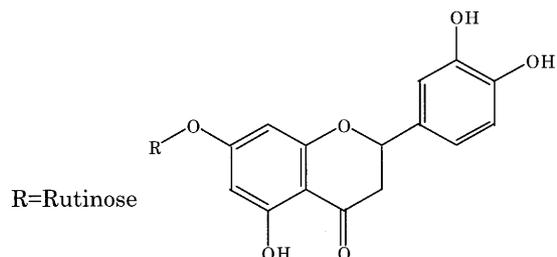
MATERIALS AND METHODS

Reagents. Sulfatase (type H-5) and β -glucosidase (G4511, from almonds) were obtained from Sigma Chemical Co., St. Louis, MO, USA. Other reagents used in this study were of analytical or HPLC grade (Wako Pure Chemical Industries, Ltd., Osaka, Japan).

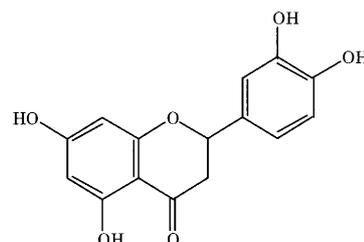
Preparation of intake sample. FG for the intake sample was prepared from lemon peel (11). Extraction from lemon peel (3 kg) was conducted using water (6 L), and the solution was applied to reversed-phase resin (Amberlite XAD-2, Rohm and Haas Co., Philadelphia). The resin was eluted with 40% ethanol and the eluate was evaporated under reduced pressure and freeze-dried to obtain a powder (248 g). FG contained 30% eriocitrin and other flavonoids (0.1% 6,8-C-diglucosyldiosmetin, 0.05% hesperidin), which were determined by HPLC analysis (11). FA for the intake sample were prepared from FG by treatment with β -glucosidase. β -Glucosidase at a final concentration of 0.1% was added to 100 mM sodium phosphate buffer solution (pH 5.0) containing 2% FG and the solution was incubated at 37°C for 4 h. It was applied to reversed-phase resin (Amberlite XAD-2). The resin was washed with water to remove carbohydrate and enzymes, and then the resin was eluted with pure ethanol. The eluate was evaporated under reduced pressure and freeze-dried to obtain a powder. FA 73.8 g was prepared from 90 g of FG with a yield of 82.2%. FA contained 14.7% eriodictyol and other flavonoids (3.0% hesperetin, 0.3% eriocitrin), which were determined by HPLC analysis (11). Lemon juice deprived of flavonoids for the intake sample was prepared by squeezing and passing the juice through resin (Amberlite XAD-2). The contents of eriocitrin and hesperidin in the juice were less than 1 mg/100 g by HPLC analysis. Vodka (40% ethanol) for the alcoholic drink was obtained from a commercial product and used for the solution of FA to contain less food components.

Subjects. Ten male volunteers, from 32 to 39 y of age (mean \pm SD, 35.9 \pm 2.6) and from 62 to 80 kg of body weight (mean \pm SD, 67.9 \pm 5.8), participated in tests 1–4. This study was carried out in accordance with the Helsinki Declaration of 1975, as revised in 1983, and approved by the Ethics Committee of Ochanomizu University. The procedures were fully explained to all the volunteers in advance, and they gave their signed informed consent before participating. In tests 3 and 4, some of the volunteers participated through the informed consent.

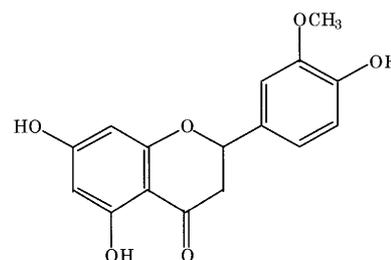
Design of intake study. Each study of the subjects was performed at intervals of more than 7 d. The subjects ingested the intake sample over a period of less than 10 min at the study site in the morning after an overnight fast. The baseline of the blood sample was obtained 10–20 min before administration. Blood samples of 10 mL were taken from subjects, and were collected into tubes containing EDTA at 0.5 h, 1 h, 2 h, and 4 h after intake of the sample by subjects. They were centrifuged at 1,000 \times g, and the plasma was fro-



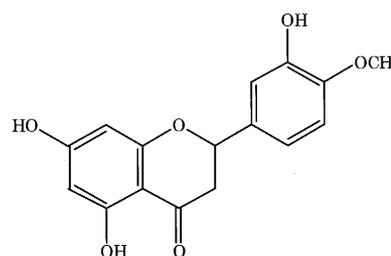
1. eriocitrin



2. eriodictyol



3. homoeriodictyol



4. hesperetin

Fig. 1. Chemical structure of eriocitrin, eriodictyol, homoeriodictyol and hesperetin.

zen at -70°C . The four intake tests were performed as follows. In test 1, ten subjects ingested 4.5 g of FG, wrapped with oblate, with 500 mL water. The content of eriocitrin in FG was 1.35 g (2.27 mmol) by HPLC analysis (11). For test 2, ten subjects ingested 3.7 g of FA, wrapped with oblate, with 500 mL water. The FA intake was estimated from the yield of FA (82.2%) prepared from the FG (4.5 g). FA was determined as 1.89 mmol eriodictyol by HPLC analysis (11). In test 3, four subjects ingested the lemon juice containing FG. FG (4.5 g) was dissolved in 500 g of the lemon juice deprived of flavonoids. For test 4, seven subjects ingest-

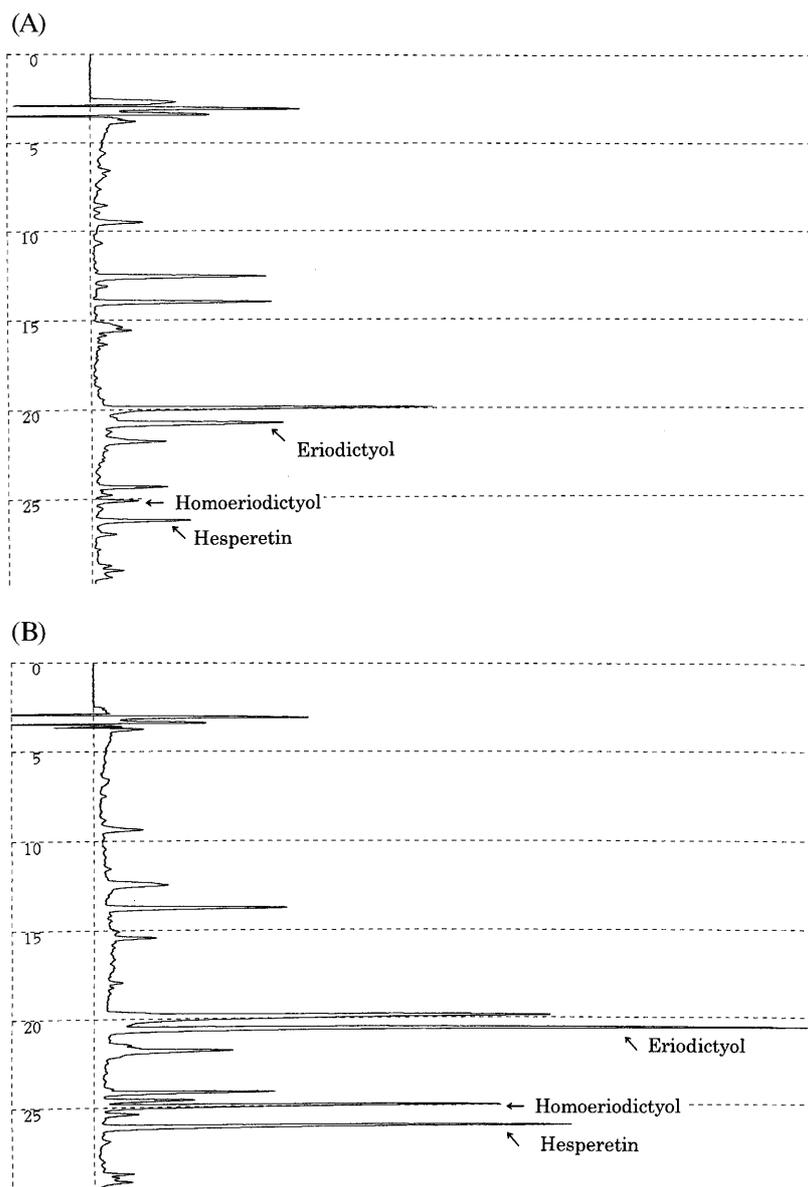


Fig. 2. HPLC profiles of plasma after ingestion of FG and EA. (A) is the HPLC profile of plasma after ingestion of FG for test 1. A plasma sample from 1.0 h after ingestion of FG (4.5 g) with 500 mL water for a subject was treated with β -glucuronidase and sulfatase. (B) is the HPLC profile of plasma after ingestion of EA for test 2. A plasma sample from 1.0 h after ingestion of FG (3.7 g) with 500 mL water for a subject was treated with β -glucuronidase and sulfatase. They were analyzed by HPLC as described in Materials and Methods.

ed 50 mL of vodka containing EA with 450 mL water. EA (3.7 g) was dissolved in 50 mL vodka.

Determination of concentration of eriocitrin metabolites in plasma. Flavanones in plasma were analyzed as previously described (24). The plasma sample (0.5 mL) was treated with 5.4×10^2 units/mL β -glucuronidase and 0.2×10^2 units/mL sulfatase for 60 min at 37°C . The mixtures were applied to a DISPO COLUMN C18H050 (Toyo Roshi Ltd., Japan). The methanol elute solution was evaporated to dryness and the residue was dissolved in 50 μL of methanol. Flavanones of eriocitrin, eriodictyol, homoeriodictyol and hesperetin (Fig. 1) in the solution were analyzed by HPLC (Shimadzu, Kyoto, Japan) using a YMC-Pack ODS column (YMC Co., Ltd., Kyoto, Japan, column size; $\phi 4.6 \times 250$ mm, particle

size; 5 μm) and UV detection (333 nm). The mobile phase contained the following: Solvent A, methanol; Solvent B, distilled water with 5% acetic acid. The initial ratio of Solvent A : Solvent B (20 : 80) was changed by a gradient toward Solvent A : Solvent B (70 : 30) for 30 min. The column temperature was maintained at 40°C and the flow rate was 1.0 mL/min. Each concentration value in the plasma was represented as mean \pm SE. The sum of three flavanones was calculated along with the sum for content of eriodictyol, homoeriodictyol, and hesperetin. The AUC (area under the blood concentration time curve) levels of eriodictyol, homoeriodictyol, hesperetin, and the sum of three flavanones were obtained from the kinetics of the values in the Figures.

Table 1. AUC_{0-4h} ($\mu\text{mol h/L}$) of metabolites in plasma after ingestion of sample.

Ingestion sample	Eriodictyol	Homoeriodictyol	Hesperetin	Sum of three flavanones
Flavonoid glycosides (FG)	5.18 \pm 0.94	1.49 \pm 0.25	5.62 \pm 0.63	12.29 \pm 1.56
Flavonoid aglycones (FA)	21.93 \pm 1.83	13.44 \pm 1.67	10.30 \pm 1.41	45.67 \pm 4.53
FG in lemon juice*	1.49 \pm 0.29	0.46 \pm 0.24	2.45 \pm 0.42	4.41 \pm 0.84
FA in vodka**	25.08 \pm 5.31	8.31 \pm 1.79	13.14 \pm 2.52	46.53 \pm 8.93

Concentration of flavonoid metabolites in plasma from 0 h to 4 h after ingestion of sample by subjects was examined and AUC level of flavonoid metabolites was calculated. Each value represents a mean \pm SE.

* Flavonoids were removed from lemon juice by resin.

** The concentration of ethanol in vodka is 40%.

RESULTS

Flavanones in plasma after ingestion of FG (4.5 g) by subjects for test 1 were analyzed by HPLC. Eriodictyol and hesperidin were not detected in plasma by treatment with or without β -glucuronidase and sulfatase. Eriodictyol, homoeriodictyol, and hesperetin were not detected in plasma without treatment of β -glucuronidase and sulfatase, but were detected by treatment with both enzymes (Fig. 2A). The respective concentrations of eriodictyol, homoeriodictyol, and hesperetin and the sum of three flavanones in plasma are shown in Fig. 3. Alteration in their plasma concentrations up to 4 h after ingestion of FG was mild.

Flavanones in plasma after a single administration of 3.7 g FA to subjects for test 2 were also analyzed by HPLC. Eriodictyol, homoeriodictyol, and hesperetin were not detected in plasma without treatment of β -glucuronidase and sulfatase, but detected by treatment with both enzymes (Fig. 2B). The concentrations of eriodictyol, homoeriodictyol, hesperetin, and the sum of three flavanones are shown in Fig. 4. Their concentrations in plasma after ingestion of FA reached a maximum peak at 1 h. These results were shown as the difference in plasma concentration alteration after ingestion of FG and FA (Fig. 3 and 4). The AUC levels of eriodictyol, homoeriodictyol, hesperetin, and the sum of three flavanones after ingestion of FA were 4.2, 9.0, 1.8, and 3.7 times higher than that after ingestion of FG, respectively (Table 1).

Furthermore, test 3 was carried out to examine the difference in plasma absorption after ingestion of FG dissolved in lemon juice. The content of eriodictyol in 4.5 g of FG corresponds to 500 g of lemon juice, which was prepared by homogenizing lemon fruits that were peeled with a knife to remove the flavedo, and was determined by HPLC analysis according to the method of Miyake et al (11). The lemon juice deprived of flavonoids was prepared to avoid the influence of hesperetin metabolized from hesperidin in plasma after ingestion of lemon juice. Flavanones in plasma for test 3 after ingestion of FG (4.5 g) dissolved in 500 g of the lemon juice without flavonoids were determined by HPLC for the purpose of examining the absorption characteristics after ingestion of FG in lemon juice. Flavanones of metabolites were not detected in plasma without treat-

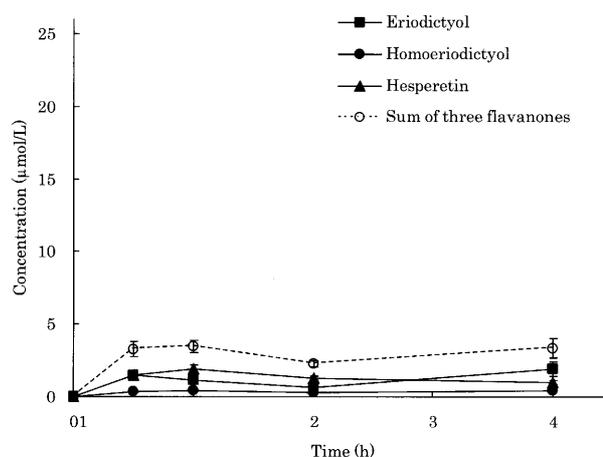


Fig. 3. The concentration of flavanones in plasma after ingestion of FG. Flavanones in plasma after ingestion of FG (4.5 g) with 500 mL water by ten subjects for test 1 were analyzed by HPLC. Each value of concentration in plasma represents the mean \pm SE ($n=10$).

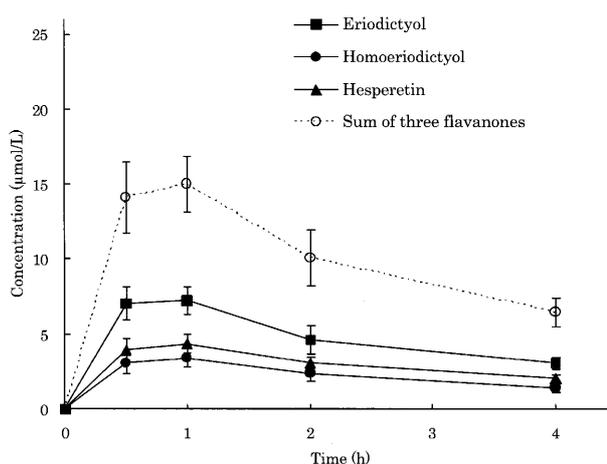


Fig. 4. The concentration of flavanones in plasma after ingestion of FA. Flavanones in plasma after ingestion of FA (3.7 g) with 500 mL water by ten subjects for test 2 were analyzed by HPLC. Each value of concentration in plasma represents the mean \pm SE ($n=10$).

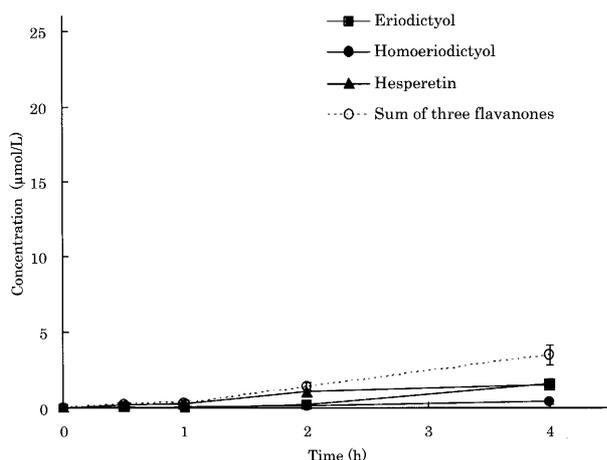


Fig. 5. The concentration of flavanones in plasma after co-administration of FG and lemon juice. Flavanones in plasma after ingestion of FG (4.5 g), which was dissolved with 500 g of the lemon juice from which flavonoids were removed, by four subjects for test 3 were analyzed by HPLC. Each value of concentration in plasma represents the mean \pm SE ($n=4$).

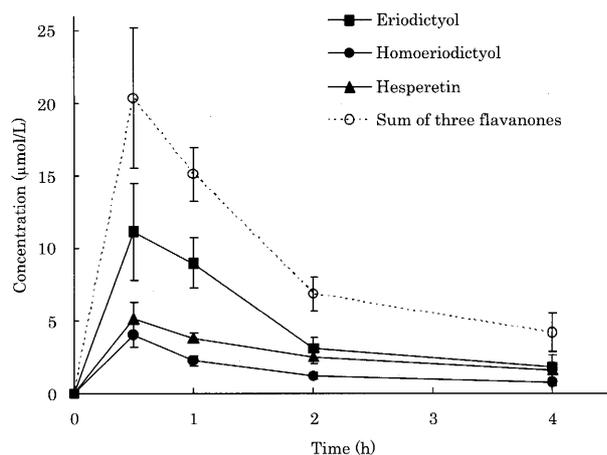


Fig. 6. The concentration of flavanones in plasma after co-administration of FA and vodka. Flavanones in plasma after ingestion of FA (3.7 g), which was dissolved with vodka (50 mL), with 450 mL water by seven subjects for test 4 were analyzed by HPLC. Each value of concentration in plasma represents the mean \pm SE ($n=7$).

ment of β -glucuronidase and sulfatase, but detected by treatment with both enzymes. Their concentrations in plasma slowly increased up to 4 h as shown in Fig. 5. The AUC level of flavanone metabolites in plasma after co-administration of FG and lemon juice was lower than that after FG (test 1) and FA (test 2), as shown in Table 1.

FG are able to dissolve completely in water and lemon juice, while FA consisting abundantly of eriodictyol did not dissolve in water but in ethanol. Since, FA dissolved in vodka (40% ethanol), the solution sample was used for test 4. Test 4 was carried out for the purpose of examining the absorption characteristics in plasma

after ingestion of FA together with alcohol. Flavanones in plasma for test 4 after ingestion of FA (3.7 g) dissolved in vodka (50 mL) with 450 mL of water were determined by HPLC. Flavanones of metabolites were not detected in plasma without treatment of β -glucuronidase and sulfatase, but detected by treatment with both enzymes. The concentration peak of flavanones in plasma was at a maximum at 0.5 h and was higher than that of FA, as shown in Fig. 6. However, the AUC level of flavanones after ingestion of FA with alcohol drink was shown to be similar to that after FA ingestion (Table 1).

DISCUSSION

It has been reported that eriocitrin is metabolized to the eriodictyol of its aglycone by intestinal bacteria in vitro (23) and that the metabolites after oral administration of eriocitrin to rats are detected in plasma as the glucuro- and/or sulfo-conjugates of eriodictyol, homoeriodictyol, and hesperetin (24). In this study, flavanones in plasma after ingestion of FG containing an abundance of eriocitrin were determined for the purpose of examining eriocitrin metabolites in humans. Flavanones of eriodictyol, homoeriodictyol, and hesperetin in plasma after ingestion of FG by subjects for test 1 were not detected without treatment of β -glucuronidase and sulfatase but detected by treatment with their enzymes as a result of HPLC analysis (Fig. 2). The result was shown to exist in plasma as the glucuro- and/or sulfo-conjugates of eriodictyol, homoeriodictyol, and hesperetin. In tests 2–4, the flavanones were also detected by treatment with β -glucuronidase and sulfatase. These flavanones have been suggested to be metabolites of eriocitrin in human plasma. Metabolites in human plasma after ingestion of citrus juice containing hesperidin or naringin are detected as glucuro- and/or sulfo-conjugates of their aglycone in plasma but not as hesperidin or naringin (21, 22). In test 1, it was shown that eriocitrin of flavanone glycoside was not detected in plasma after ingestion of FG consisting abundantly of eriocitrin, and that glucuro- and/or sulfo-conjugates of its aglycone existed in plasma. The metabolic process of eriocitrin in humans was suggested to be similar to those of hesperidin and naringin in citrus juice. It was suggested that eriodictyol was absorbed from the intestine after eriocitrin was converted to eriodictyol by deglycosylation of intestinal bacteria (23) or small intestinal epithelial cell β -glucosidase (25–26). Then, its metabolism was suggested to evolve into the methylation by catechol-*O*-methyltransferase (27) and the conjugation with glucuronic acid and/or sulfonic acid.

Flavonoids are widely distributed in foods and beverages of plant origin in the form of glycosides, although they are occasionally found as aglycones (3, 4). Flavonoids of lemon fruit were mainly glycosides such as eriocitrin and hesperidin but their aglycones do exist in small amounts (11). However, eriodictyol as aglycone was reported to have higher antioxidative activity than eriocitrin as its glycoside (9, 11). In this study, the absorption characteristics of FA consisting abundantly

of eriodictyol were examined because FA containing eriodictyol were thought to be important as functional food materials. The difference in bioavailability between glycosides and aglycones of resveratrol and quercetin after oral ingestion of grape juice preparations or pure aglycones in humans has revealed that the aglycone forms are absorbed to a higher extent than their glycosides in grape juice (18). Furthermore, soy isoflavone aglycones containing genistein and daidzein have been reported to be absorbed faster and in five times higher amounts than their glucosides in human plasma (19). In this study, absorption of FA was shown to be faster and higher than that of FG (Fig. 3, Fig. 4, Table 1), because eriodictyol had no sugar moiety and did not need to be deglycosylated.

Furthermore, plasma flavanone metabolites after ingestion of FG which were dissolved in lemon juice or FA which was dissolved in alcohol drink were examined for absorption characteristics in co-existing solutions of FG and FA. In Fig. 5, it was suggested that FG were absorbed slowly and in low amounts up to 4 h in the presence of lemon juice components not containing flavonoids. The determination of flavanones in plasma after ingestion of grapefruit juice and orange juice had been reported (20–22). After ingestion of grapefruit juice containing an abundance of naringin, the concentration of naringenin in plasma detected by treatment with β -glucuronidase and sulfatase reached a peak at 4 h (21). After ingestion of orange juice containing an abundance of hesperidin, the concentration of hesperetin in plasma detected by treatment with β -glucuronidase and sulfatase reached a peak between 5 h and 7 h (22). The concentration peak of flavanones in plasma after ingestion of FG dissolved in 500 g of the lemon juices deprived of flavonoids may be more than 4 h. The AUC of metabolites in plasma up to 4 h after ingestion of FG dissolved in lemon juice was changed to a low level in comparison with that after FG ingestion with water by the influence of lemon juice (Table 1).

In test 4, FA dissolved in alcohol were shown to have the effect of hastening the absorption of eriodictyol (Fig. 6). The result was suggested to be influenced by the solubilization of eriodictyol. However, the AUC of metabolites in plasma after ingestion of FA with an alcoholic drink for test 4 was similar to that after ingestion of FA with water for test 3, and there was no change in the absorption amount of eriodictyol (Table 1). It was reported that the concentration of (+)-catechin in plasma after ingestion of dealcoholized red wine reconstituted to its original volume with either aqueous ethanol to contain 13% ethanol or water in humans was not significantly different (28). Ethanol of co-existence solution for flavonoids was speculated not to affect in the amount of absorption of flavonoids.

As for the ingestion of FA for tests 2 and 4, eriodictyol had the highest AUC level of flavanones (Table 1). After ingestion of FG with water for test 1, the AUC level of eriodictyol was similar to that of hesperetin. After ingestion of FG with lemon juice for test 3, AUC level of eriodictyol was lower than that of hesperetin. From these

results, the high concentration of eriodictyol in plasma was suggested to relate to the fast and high amounts of absorption of eriodictyol.

Flavanone glycosides are abundant in a wide variety of citrus fruit and are known for their functional activity such as antioxidative activity (5, 6). The functional activity and metabolic process of eriocitrin, particularly abundant in lemon fruit, have been investigated (23, 24). This study examined the absorption characteristics of flavanone glycosides and their aglycones in lemon fruit through determination of flavanones in plasma after ingestion of FG and FA in humans. Aglycones were shown to be absorbed faster and in higher amount than their glycosides and to be absorbed more quickly in the presence of alcohol. It is expected that the absorption characteristics of lemon flavonoids in human plasma will lead to an understanding of the physiological function of lemon flavonoids such as eriocitrin and eriodictyol.

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