Studies on absorption, metabolism, distribution and excretion of black soybean polyphenols and their effects on vascular function

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Studies on absorption, metabolism, distribution and excretion of black soybean polyphenols and their effects on vascular function

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Abbreviation

8-OHdG	8-hydroxy-2'-deoxyguanosine
AAPH	2,2'-azobis-2-methyl-propanimidamide dihydrochloride
ALP	alkaline phosphatase
ALT	alanine aminotransferase
APG	accelerated plethysmogram
AST	aspartate aminotransferase
$AUC_{(0\rightarrow 24\ h)}$	area under concentration–time curve from 0 to 24 h
$AUC_{(\infty)}$	area under concentration–time curve from 0 to infinity
AUC_{extrap}	area under concentration–time curve extrapolated from 24 h to infinity
BE	black soybean seed coat extract
BHT	2,6-bis(1,1-dimethylethyl)-4-methylphenol
BMI	body mass index
BUN	blood urea nitrogen
C_{max}	maximum plasma concentration
CK	creatine kinase
CVD	cardiovascular diseases
DHPAA	3,4-dihydroxyphenylacetic acid
DP	degree of polymerization
EDTA	ethylenediaminetetraacetic acid
eGFR	estimated glomerular filtrationrate
FMD	flow-mediated dilation
GLUT4	glucose transporter 4
GSIS	glucose-stimulated insulin secretion
γ-GTP	γ- glutamyltranspeptidase

Hb	hemoglobin
HDL-C	high-density lipoprotein cholesterol
HEL	hexanoyl-lysine
HPLC	high performance liquid chromatography
HPLC-FLD	HPLC coupled with a fluorescence detector
HPLC-UV	HPLC coupled with a UV detector
HPPA	3-hydroxyphenylpropionic acid
HRQOL	health-related quality of life
Ht	hematocrit
LC-MS/MS	liquid chromatograph-tandem mass spectrometer
LDL-C	low-density lipoprotein cholesterol
LDH	lactate dehydrogenase
LOD	limit of detection
LOQ	limit of quantification
MCV	mean corpuscular volume
MCH	mean corpuscular hemoglobin
MCHC	mean hemoglobin concentration
MDA	malonyldialdehyde
MPO	myeloperoxidase
NO	nitric oxide
ORAC	oxygen radical absorbance capacity method
PFA	perfluoroalkoxy alkanes
PI3K	phosphoinositide 3-kinase
PLT	platelet
PWV	pulse wave velocity
RBC	red blood cells
SDS	sodium dodecyl sulfate

SF-36	36-item Short-Form Health Survey
SPE	solid phase extraction
$t_{1/2}$	plasma half-life
TBA	Thiobarbituric acid
TBARS	thiobarbituric acid reactive substances
TG	triglycerides
T_{max}	time for flavan-3-ols to reach their maximum plasma concentration
TMP	1,1,3,3-Tetramethxypropane
TP	total protein
Trolox	6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid
WBC	white blood cells

Chapter 1. General introduction

Vascular function and dietary polyphenols

Vascular function is closely related to the risk of cardiovascular diseases (CVD) [1-3]. Aging process can result in vascular dysfunction [4], and it has been identified as the dominant risk factor [5-6]. It causes functional and structural changes of the vascular wall. Increase in the vascular stiffness is the major symptom, and it compromises vascular adaption to blood flow and pressure changes. Oxidative stress is another important trigger of vascular dysfunction, and the connection of oxidative stress with the aging process is widely accepted [7], indicating that decline in vascular function is an inevitable result of aging. Alteration of nitric oxide (NO) is a key molecular of the underlying mechanism [8]. Releasing of NO by endothelial cells decreases the intracellular concentration of calcium, causes relaxation of vascular smooth muscle as a potential vasodilator. However, aging and oxidative stress quenches the production of NO, hampers the NO-mediated responses and eventually leads to the vascular dysfunction [9]. Polyphenols are natural substances present in beverages obtained from plants, fruits and vegetables. It is reported that polyphenols possess the dynamic capacity to protect against age-associated disorders [10]. Epidemiological data suggests that a diet high in polyphenol-rich food is associated with lower CVD risk by protective effects on the vascular function [11-13]. However, it remains unclear if this association reflects a cause-and-effect of polyphenols improving vascular function.

Black soybean polyphenols

Black soybeans (*Glycine max*) are soybeans that have black seed coat on the outside of the beans. It is originated from Asia and has been consumed as a health food and folk medicine for many centuries [14]. Similar as the yellow soybeans, black soybeans are rich in protein, lipids, starch, minerals and polyphenolic compounds.

Commonly, isoflavones and proteins in the cotyledons of black soybeans are considered as the primary health beneficial components in black soybeans [15, 16]. Recently, black soybean polyphenols have raised interest because researches showed that black soybeans has the greatest antioxidant efficiency compared to other colored soybeans [17, 18], and the characteristic antioxidant potential is due to the presence of anthocyanidins and flavan-3-ols (chemical structures were shown in Figure G1.) in its seed coat [19-25]. Studies have reported that black soybean polyphenols contribute to lowering the risk of lifestyle-related diseases and improving the pathological conditions, such as anthocyanidins reducing the blood pressure and inhibiting the activity of collagen-induced platelet aggregation [26, 27], while flavan-3-ols improving vascular function [28].

Nevertheless, the amount of black soybean consumption is still small worldwide, since the consumption of soybeans by human is still a relatively small portion of total use compared to the use for livestock feed, cooking oil and biodiesel [29]. Fortunately, the consumption of soybean food and drink products have increased in popularity because of its nutritional value, and the share of consumers who reported eating or drinking soy products in 2016 has grown nearly 8 percent from 2011 [30]. Therefore, there is a great potential for black soybean consumption to grow as long as their nutritional value be more realized.

Limitations of current studies on the black soybean polyphenols

Bioavailability of flavan-3-ols is not fully understood yet and it is the most challenging issue for applying black soybean polyphenols as chemoprevention agent. It is generally agreed that polyphenols have low oral bioavailability [31]. Cyanidin-3-*O*-glucoside is the main anthocyanidin in black soybean polyphenols [23]. Its bioavailability has been frequently reported by animal and human studies indicating

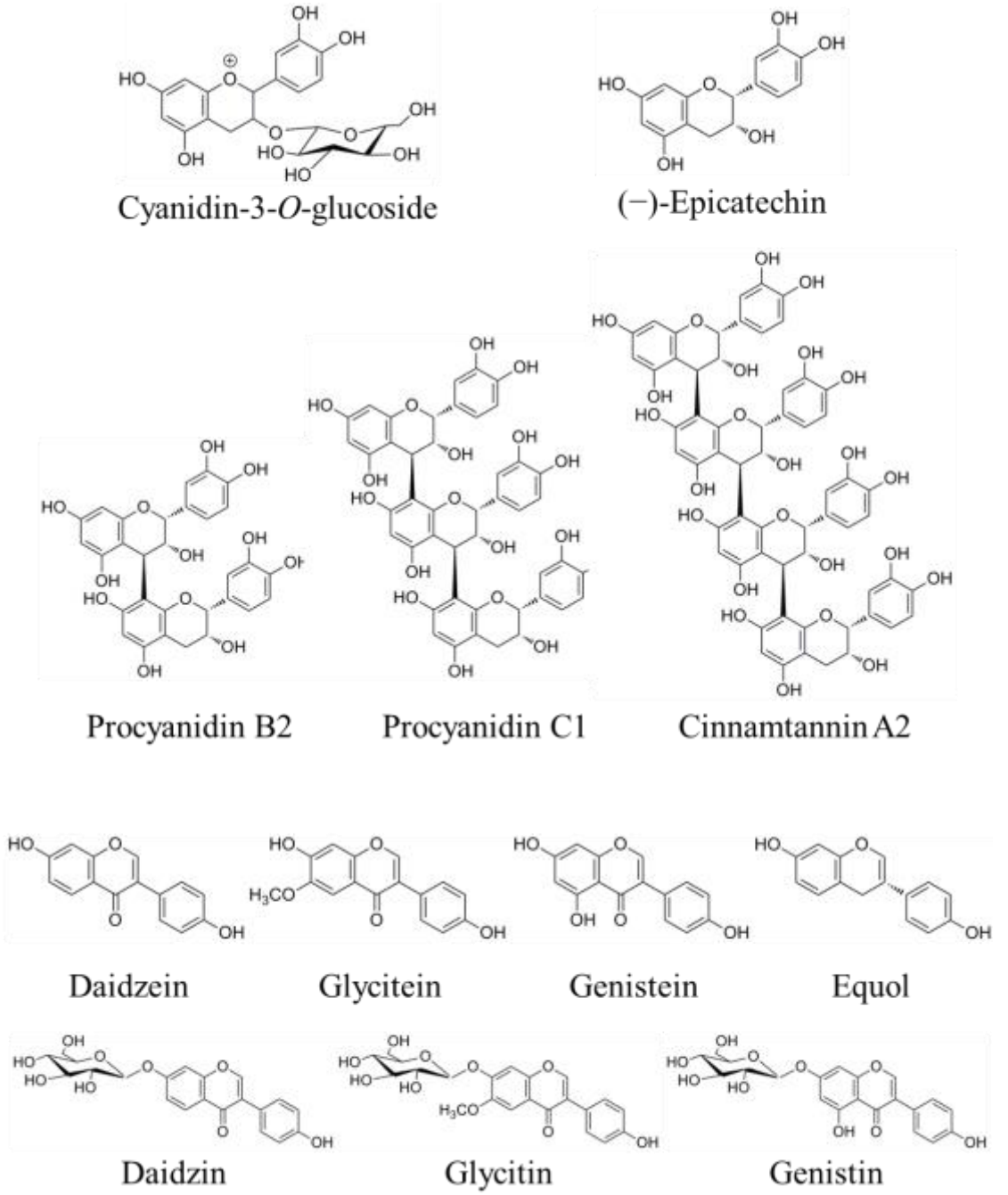


Figure G1. Chemical structure of major polyphenol compounds in black soybean

cyanidin-3-*O*-glucoside is absorbed mainly in their intact glycoside form and rapidly reach the circulatory system [32]. Conflicting researches are also reported suggesting the majority of absorbed anthocyanins are present as lower molecular weight products of microbial degradation [33]. Bioavailability of isoflavones has also been reported by many studies, suggesting daidzin, genistin and glycitin (major isoflavones in soybeans) are broken down into their aglycones, namely daidzein, genistein and glycitein respectively, by β-glucosidases before entering the circulatory system [34]. Moreover, daidzein are furtherly metabolized into equol by bacterial flora in the intestine [35]. Compared to the bioavailability of anthocyanidin and isoflavone, current information on flavan-3-ols bioavailability, especially procyanidins, is inadequate and conflicting [36].

Another issue is derivate from the bioavailability. Even with low oral bioavailability, these polyphenols are still reported showing remarkably beneficial effects on lifestyle-related diseases, indicating an inconsistency between their observed effects and the bioavailability. However, only few researches linked the bioavailability of polyphenols with their observed beneficial effects [37-38]. Therefore, more detailed research elucidating the bioavailability of dietary polyphenols, as well as measuring their related beneficial effects are needed.

In addition, to eventually develop the black soybeans as a functional food that can actually exert beneficial effect to human health, intervention studies are needed. It is reported that BE supplementation (2.5 g/day) for 8 weeks improved visceral fat and plasma lipid profiles in overweight Korean adults [39], consisting with the anti-obesity and hypolipidemic effects of black soybean anthocyanidins observed in the rats [40]. The effect of black soybean polyphenols on the vascular function of human was first investigated in this dissertation.

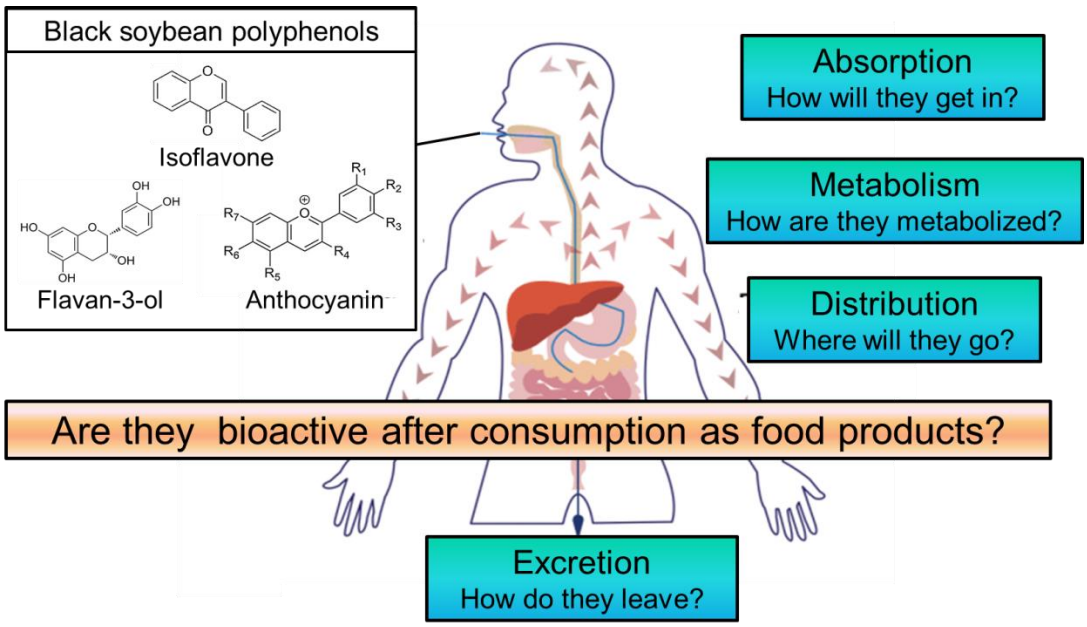


Figure G2. Limitations of the current studies on the beneficial effects of black soybean polyphenols on lifestyle-related diseases mentioned in this dissertation.

Aim of this dissertation

This dissertation aimed to investigate the absorption, metabolism, distribution and excretion of black soybean polyphenols using animal experiment, and explore its beneficial effects on the vascular function in human. In the next chapter (Chapter 2), considering the limitations of current studies on polyphenol bioavailability, the development of a new analysis method for black soybean flavan-3-ols, using high performance liquid chromatography (HPLC) coupled with fluorescence detector and diode array detector was described. Sensitivity of this analytical method for flavan-3-ols is 1000 times higher than that of a conventional HPLC method coupled with the UV detector. This analytical method was applied for the following studies on the bioavailability and physiological functions of black soybean polyphenols. In Chapter 3, using the developed HPLC method, the absorption, metabolism, distribution and faecal excretion of flavan-3-ols in black soybean seed coat, namely (−)-epicatechin, procyanidin B2, procyanidin C1 and cinnamtannin A2, was investigated. Black soybean

seed coat extract (BE) was orally administrated to male ICR mice at a dose of 250 mg/kg body weight. Mice were sacrificed within 24 h after the administration. Plasma, liver, kidney, small intestine, heart, muscle, mesenteric fat, perirenal fat, epididymal fat and feces during 0-4 h, 4-8 h, 8-12 h, 12-24h were collected for quantitative analysis of flavan-3-ols. A portion of the flavan-3-ols in BE was absorbed from the small intestine after oral administration. In the plasma, absorbed flavan-3-ols existed mainly as conjugates. In the tissues, flavan-3-ols were distributed widely and primarily in their free forms. Conjugation of flavan-3-ols occurred mainly in the small intestine, rather than in the liver. (−)-Epicatechin had the highest bioavailability, followed by procyanidin B2, procyanidin C1 and cinnamtannin A2. Moreover, BE showed significant antioxidative efficiency in the liver. In Chapter 4, the effect of roasted black soybean consumption with an intake of 30 g/day for 8 weeks on the vascular function and oxidative stress associating with polyphenols in the plasma and urine was investigated using an open-label trial in human. Lowered vascular age was observed in 33 out of 44 volunteers who finished the 8-week trial. Significantly improved vascular stiffness, increased NO, decreased 8-OHdG, HEL and MPO were also observed, indicating that vascular function and oxidative stress was improved by black soybean consumption. In addition, physiological concentrations of 12 polyphenols increased significantly in the plasma and urine, and the increase was found positively correlated to the improved vascular function and oxidative stress, indicating black soybean consumption contributed to these functions by increasing the polyphenol concentrations in human. In Chapter 5, overall discussion from the results of this dissertation and future plan is described.

Chapter 2. An analysis method for flavan-3-ols using high performance liquid chromatography coupled with a fluorescence detector

2.1. Introduction

Flavan-3-ols are the most common group of flavonoids in plant-derived foods and beverages. They possess beneficial effects, including strong anti-oxidative, anti-carcinogenic, anti-obese and anti-diabetic effect [41-43]. Food material that is rich in flavan-3-ols are also reported showing beneficial effects on health: for example, oral administration of a cacao liquor extract facilitating glucose uptake and improving postprandial hyperglycemia by promoting GLUT4 translocation *in vitro* and *in vivo* [44]; and lotus seedpods significantly inhibited growth of melanoma in C57BL/6J mice, with decreasing tumor weight by ~55% [45].

However, the oral bioavailability of flavan-3-ols is not yet clear. Lack of proper analytical method is one of the major issues. Due to the complexity of their chemical structures, oligomeric flavan-3-ols, also known as procyanidins are difficult to fully characterize. In many previous studies on the bioavailablility of flavan-3-ols, the specific chemical structures of flavan-3-ols from food materials were not characterized [46-48], causing the bioavailability of specific flavan-3-ols remains unclear. Moreover, analytical method greatly affects the limit of determination of flavan-3-ols in biological samples. In a study, procyanidin dimers and trimers in the aglycone form were detected by liquid chromatograph-tandem mass spectrometer (LC-MS/MS), and reached maximum concentrations in plasma of 0.57 nM and 0.03 nM respectively, 1 h after ingestion of 1 g/kg body weight grape seed procyanidin extract to Wistar rats [49]. In another study using the same animals and dose of the extract with different extraction method, maximum plasma concentrations of corresponding procyanidins were reported as 2.4 μM and 8.55 μM respectively, 2 h after ingestion [50]. The recovery of flavan-3-ols from biological samples was not considered in either case causing the great

difference in maximum concentrations of the same compounds in the plasma and underestimated bioavailability. Therefore, a validated analytical method with proper evaluation is needed for detection of flavan-3ols in biological samples to elucidate the bioavailability of black soybean flavan-3-ols.

Chromatographic separation of flavan-3-ols using HPLC is widely used and has become a standard analysis method [51]. Many practical separation methods have been reported for separating flavan-3-ols from food materials basing on their molecular weight or hydrophobicity [52-54]. These methods are usually coupled with mass spectrometry instruments to identify flavan-3-ols from their fragmentation patterns [55]. Main disadvantage of these methods is that fragmentation patterns of flavan-3-ols, such as procyanidin compounds, show no differences among isomers having the same degree of polymerization (DP). Meanwhile, HPLC using fluorescence detection of flavan-3-ols has the potential benefits of high sensitivity and reducing interfering signals [56]. Therefore, a highly sensitive analysis method for flavan-3-ols was developed in this chapter using HPLC coupled with a fluorescence detector (HPLC-FLD).

2.2. Material and methods

2.2.1. Reagents & materials

(+)-Catechin (≥ 98.9%) and (−)-epicatechin (≥ 98.9%) were purchased from Kurita Analysis Service Co. Ltd (Ibaraki, Japan). Authentic compounds of procyanidin B2, procyanidin C1, cinnamtannin A2 and ChronoCare®, a commercially available BE, were obtained from Fujicco Co., Ltd (Kobe, Japan). Procyanidin B3-OAc, used as internal standard, was synthesized and purified by solid phase extraction [57]. Chemical structure of flavan-3-ols in the BE and procyanidin B3-OAc was shown in Figure G1. HPLC grade methanol, acetonitrile, formic acid and phenol reagents for Folin–Ciocalteu method were obtained from Wako Pure Chemical Industries (Osaka, Japan).

β-Glucuronidase from *E. coli.,* (type IX-A) was purchased from Sigma-Aldrich Co. LLC (St. Louis, USA). All other reagents and chemicals were of analytical grade, unless otherwise stated.

2.2.2. Equipment

HPLC was performed using a system equipped with a DGU-20A 3R degas unit, LC-20AD XR binary pump, SIL-20AC XR auto sampler, RF-20A XS fluorescence detector, SPD-M20A diode array detector, CTO-20AC column oven and CBM-20A communications bus module connected to a LC work station (Shimadzu Corporation, Kyoto, Japan). A Cadenza CL-C18 column (φ 250 mm × 4.6 mm, 3 μm, Imtakt, Kyoto, Japan) was selected and a guard column (Cadenza CL-C18, φ 5 mm × 2 mm, 3 μm, Imtak, Kyoto, Japan) was used to protect the analytical column.

2.2.3. Chromatographic conditions

Formic acid solution (1:1000, v/v, formic acid: MilliQ water) was used as mobile phase A and acetonitrile was mobile phase B. Separation of flavan-3-ols was achieved using a linear gradient from 5–15% B over 0–45 min; 15–80% B over 45–50 min; 80% B, over 50–53 min; 5% B, over 53–70 min. The gradient from 53 min onwards was set for re-equilibration between samples. The flow rate was set at 0.7 ml/min with an injection volume of 10 μl and column temperature maintained at 40 °C. Fluorescence of the procyanidins was measured by excitation at 276 nm and emission was monitored at 316 nm. Absorbance of the procyanidins was monitored at 280 nm using a UV detector.

2.2.4. Limit of detection and limit of quantification

All authentic compounds were dissolved in 50% methanol to form 1 mM stock solutions and stored at −20 °C until analysis. The limit of detection (LOD) was defined as the absolute amount of analyte that maintained a signal-to-noise ratio (peak height) of

3:1, while the limit of quantification (LOQ) was defined as the absolute amount of analyte that produced a signal-to-noise ratio of 10:1. Noise was the magnitude of background response, which was determined by analyzing blank samples (50% methanol alone).

2.2.5. Calibration curves

A series of standard solutions of (+)-catechin, (−)-epicatechin, procyanidin B2, procyanidin C1 and cinnamtannin A2 across a concentrations range of 0.001 to 10 μM were prepared. The linearity of each calibration curve was determined by plotting the peak area ratio of authentic procyanidin compounds to an internal standard, procyanidin B3-OAc. A linear regression method was used to determine the slope and correlation coefficient of the linear regression equation.

2.2.6. Preparation of BE

Black soybeans cultivated under three different fertilization conditions were analyzed, namely, conventional farming, basal manure application and intertillage. Conventional farming involved cultivation with a commercially available organic fertilizer, Zubariyuuki®, which was applied to the soil before seeding. Basal manure application involved cultivation with an organic fertilizer, derived from fermented rice bran, oil cake, fish powder and crushed oyster shell, applied to the soil at 0.06 kg/m^2 before seeding. Intertillage also involved cultivation with the same fermented organic fertilizer (0.06 kg/m^2), which was applied to the soil during the growth of the black soybeans. For analysis of procyanidin content of the BEs, the extraction was performed according to a previous report by Ito C. *et al.* [25] with some modifications. Briefly, 0.5 g of black soybean seed coat was extracted with 40 ml acetone: water: formic acid (70:29.5:0.5, v/v/v) 3 times. The extraction solvent (about 120 ml) was evaporated using a rotary evaporator and the concentrated extract was transferred to an amber vial. The

extract was then evaporated to dryness under a stream of nitrogen gas in a fume hood. Each extract was re-dissolved in 50% methanol to a concentration of 30 mg/ml to prepare the stock solutions, which were preserved at −20 °C until analysis.

2.2.7. Analysis of BE

To estimate the content of (+)-catechin, (−)-epicatechin, procyanidin B2, procyanidin C1 and cinnamtannin A2 in the BE, the stock solution of each authentic compound was diluted to a concentration of 0.1 mg/ml and analyzed. The content was calculated and expressed in mg/g BE based on the calibration curve. The total polyphenol content in the BE was measured by the Folin–Ciocalteu method [58] with some modifications. Briefly, 0.5 ml of the 0.1 mg/ml BE solution was added to 2.5 ml of 0.2 N phenol reagent and mixed in glass test tube. After incubation for exactly 3 min, 2.5 ml of 10% (w/v) Na_2CO_3 solution was added to terminate the reaction. After 1 h, the absorbance was measured at 750 nm. Gallic acid was used as a standard compound over a concentration range of 0.025 to 0.1 mg/ml. The total polyphenol content was calculated and expressed as g gallic acid equivalent/g BE. The antioxidant capacity of the BE was measured using oxygen radical absorbance capacity method (ORAC) [59]. All reagents were dissolved and diluted in 75 mM potassium phosphate buffer (pH 7.4). A 20 μl aliquot of diluted sample was mixed with 120 μl of 70 nM fluorescein and added to a 96-well plate. After incubation at 37 °C for 15 min, 60 μl of 12 mM 2,2'-azobis-2-methyl-propanimidamide dihydrochloride (AAPH) was quickly added to the mixture for the following detection. The total volume of the reaction mixture was miniaturized to 200 μl. To make a calibration curve, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox) was used as a standard compound over a concentrations range of 0 to 1 mM. The fluorescence intensity was measured every 2 min over 90 min by a Wallac Multilabel Counter (Avro SX, PerkinElmer, USA) at excitation and emission wavelengths of 485 nm and 535 nm,

respectively. The oxidant capacity of the BE was measured and expressed as μmol trolox equivalent/g BE according to the calibration curve.

2.2.8. Animal experiment

2.2.8.1. Experimental design

Animal experiments in this study were approved by Institutional Animal Care and Use Committee (27-05-09) and carried out according to the Guidelines of Animal Experimentation of Kobe University. Male ICR mice (9 weeks old, n= 9) were obtained from Japan SLC (Shizuoka, Japan). They were maintained at 22±3 °C under an automatic lighting schedule (8:00 am–8:00 pm). The mice were given free access to tap water and commercial chow (Rodent lab diet EQ from Japan SLC). After acclimatization for 1 week, ICR mice were randomly divided into 2 groups: control (n=3) and BE groups (n=6). The BE group were orally administrated ChronoCare® at a dose of 250 mg/kg body weight under fasting condition, while the control group were given the same volume (200 μl) of distilled water as a vehicle. One hour after administration, the mice were sacrificed by drawing blood from cardiac puncture under anesthesia using pentobarbital. Plasma was isolated from blood by centrifugation at 3,000 g for 10 min at 4 °C and stored immediately at −80 °C until analysis.

2.2.8.2. Recovery and flavan-3-ols in the plasma

To evaluate the loss during extraction process, recovery was estimated by spiking mouse plasma taken from the control group with a known amount of ChronoCare®. For the extraction process, an aliquot of 120 μl plasma mixed with 10 μl of 1 mM ascorbic acid, which was added to prevent oxidation of procyanidins during extraction, was added to a PFA tube (15 ml, Savillex, USA). β-Glucuronidase (500 U/sample) from *E. coli.* (type IX-A) was used for de-conjugation. After 1 h-incubation at 37 °C, 1 ml of

acetonitrile was added to the mixture to terminate the reaction and extract the flavan-3-ols. The mixture was sonicated for 5 min and centrifuged at 3000 g for 10 min. The supernatant was collected, and the extraction step was repeated two more times. Pooled supernatant was evaporated to dryness under a nitrogen steam in a fume hood. Dried material was dissolved in 50 µl of 50% methanol and applied to HPLC.

2.3. Results and discussion

2.3.1. Method validation

Standard compounds were analyzed by HPLC to identify flavan-3-ols. Retention times of (+)-catechin, (−)-epicatechin, procyanidin B2, procyanidin C1 and cinnamtannin A2 were 20.4, 28.9, 24.9, 34.3, and 37.7 min, respectively. Procyanidin B1 and (−)-epicatechin-(4β→6)-(−)-epicatechin-(4β→8)-(−)-epicatechin-(4β→8)-(−)-epicatechin (referred to as procyanidin 4-1 in this experiment), which are isomers of procyanidin B2 and cinnamtannin A2, respectively, were also analyzed to investigate the separation of isomers (Figure 2.1). As shown in Table 2.1, the LOD of (+)-catechin, (−)-epicatechin, procyanidin B2, procyanidin C1 and cinnamtannin A2 detected by HPLC-UV were 1.7, 2.9, 25.0, 40.0 and 30.0 ng, respectively. Similarly, the LOD values for these compounds detected by HPLC-FLD were 3.0×10^{-3}, 4.0×10^{-3}, 14.0×10^{-3}, 18.5×10^{-3} and 23.0×10^{-3} ng, respectively. The LOQ of (+)-catechin, (−)-epicatechin, procyanidin B2, procyanidin C1 and cinnamtannin A2 detected by HPLC-UV were 7.7, 14.5, 40.0, 150.0 and 500.0 ng, respectively. The LOQ detected by HPLC-FLD were 10.0×10^{-3}, 29.0×10^{-3}, 28.5×10^{-3}, 54.1×10^{-3} and 115.0×10^{-3} ng, respectively. The correlation coefficients R^2 of all calibration curves were ≥0.999 (Table 2.2).

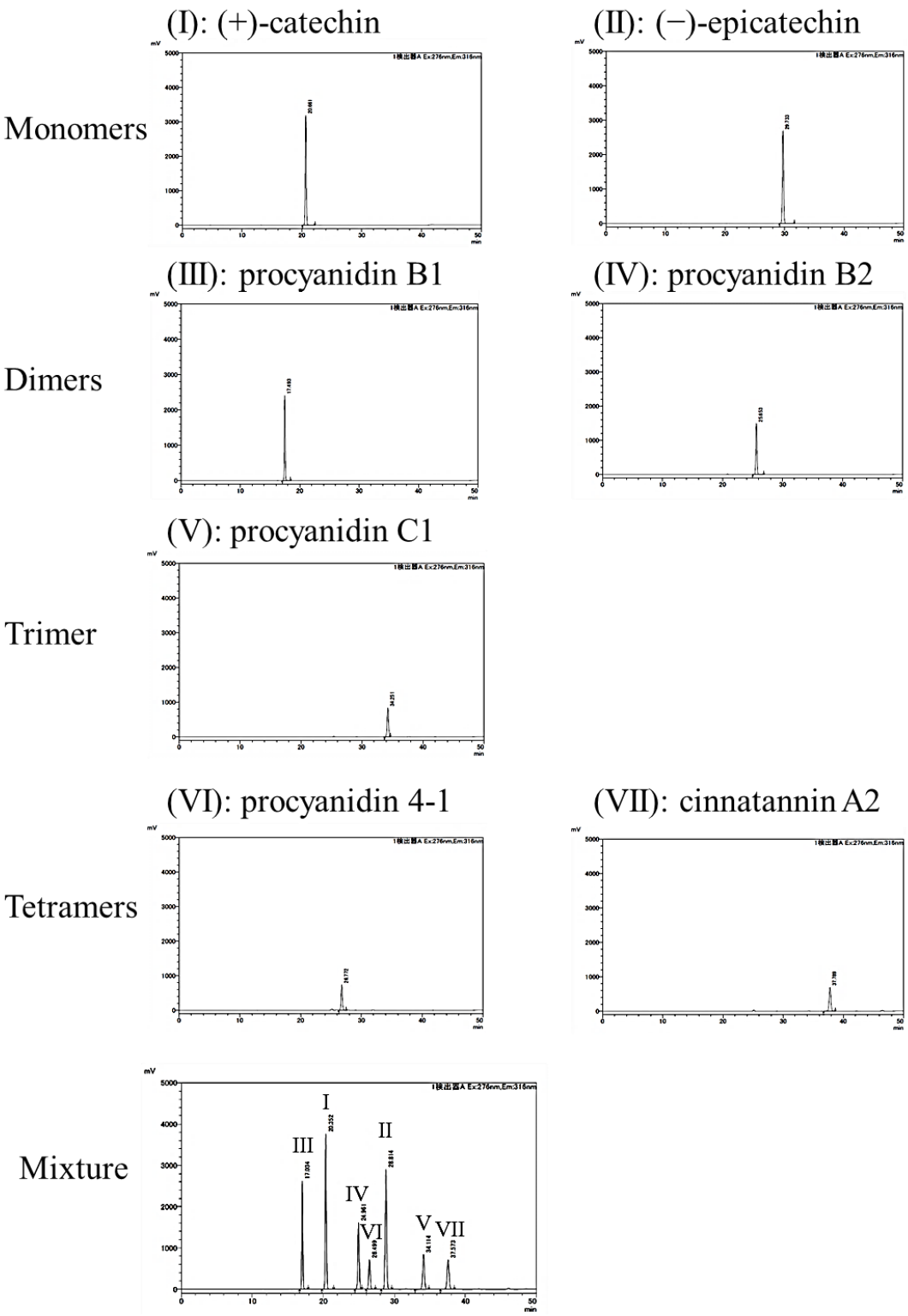


Figure 2.1. Typical chromatogram of flavan-3-ols. Peak identification: (I) (+)-catechin, (II) (−)-epicatechin, (III) procyanidin B1, (IV) procyanidin B2, (V) procyanidin C1, (VI) procyanidin 4-1, (VII) cinnamtannin A2.

Table 2.1. Limit(s) of detection (LOD) and limit(s) of quantification (LOQ) of flavan-3-ols detected by UV and fluorescence detectors

Flavan-3-ols	LOD[a] (ng)		LOQ[b] (ng)	
	HPLC-UV[c]	HPLC-FLD[d]	HPLC-UV[c]	HPLC-FLD[d]
(+)-Catechin	1.7	$3.0x10^{-3}$	7.7	$10.0x10^{-3}$
(−)-Epicatechin	2.9	$4.0x10^{-3}$	14.5	$29.0x10^{-3}$
Procyanidin B2	25.0	$14.0x10^{-3}$	40.0	$28.5x10^{-3}$
Procyanidin C1	40.0	$18.5x10^{-3}$	150.0	$54.1x10^{-3}$
Cinnamtannin A2	30.0	$23.0x10^{-3}$	500.0	$115.0x10^{-3}$

a. LOD is defined as the absolute amount of analyte when its signal-to-noise ratio (S/N) is 3.
b. LOQ is defined as the absolute amount of analyte when its signal-to-noise ratio (S/N) is 10.
c. Absorbance monitored at 280 nm.
d. Excitation at 276 nm and emission monitored at 316 nm.

Table 2.2. Analytical parameters of flavan-3-ols by HPLC with fluorescence detector

Flavan-3-ols	Retention time (min)	R^2	Linearity (μM)	Calibration curve
(+)-Catechin	20.4	0.999	0.001-10.000	y=0.6233x
(−)-Epicatechin	28.9	0.999	0.001-10.000	y=0.6104x
Procyanidin B2	24.9	0.999	0.001-10.000	y=0.5930x
Procyanidin C1	34.3	0.999	0.001-10.000	y=0.4665x
Cinnamtannin A2	37.7	0.999	0.001-10.000	y=0.3609x

y is represented as the peak area ratio of flavan-3-ol and internal standard, and x is represented as concentration, μM.

2.3.2. Application of HPLC-FLD

2.3.2.1. Flavan-3-ol contents in black soybeans grown under three different conditions

HPLC-FLD was applied to analyze flavan-3-ol contents in black soybean seed coat (Figure 2.2). For soybeans grown by conventional farming, (+)-catechin, (−)-epicatechin, procyanidin B2, procyanidin C1 and cinnamtannin A4 content in the seed coat were 0.17, 21.34, 10.55, 7.31 and 5.95 mg/g BE, respectively. For the BE from soybeans grown with basal manure application, the contents were 0.20, 22.84, 11.62, 8.50 and 7.32 mg/g extract, respectively; and the seed coat of soybeans grown under intertillage conditions had contents of 0.22, 24.63, 12.44, 8.83 and 7.51 mg/g extracts, respectively. The amount of flavan-3-ols from the three BEs decreased in

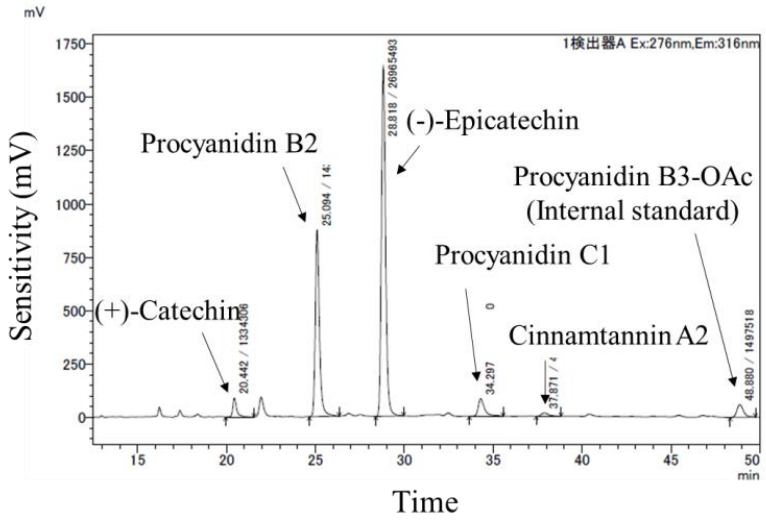


Figure 2.2. Typical chromatogram of 0.1 mg/ml BE.

the order of intertillage > basal manure application > conventional farming (Figure 2.3). The total polyphenol contents and antioxidant capacity measured by the Folin–Ciocalteu method and ORAC also followed a similar trend (Figure 2.4). It was noteworthy that the BE antioxidant capacity of soybeans grown under intertillage conditions was significantly higher than that of soybeans grown by the conventional farming method. This indicates that fertilization methods affected polyphenol content and antioxidant capacity

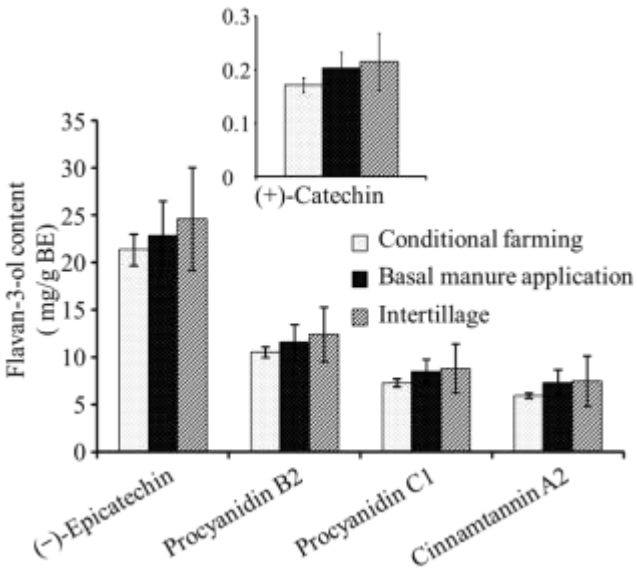


Figure 2.3. Flavan-3-ol contents in black soybean seed coat cultivated by three different fertilization methods. The amounts of flavan-3-ols are represented as mg/g BE.

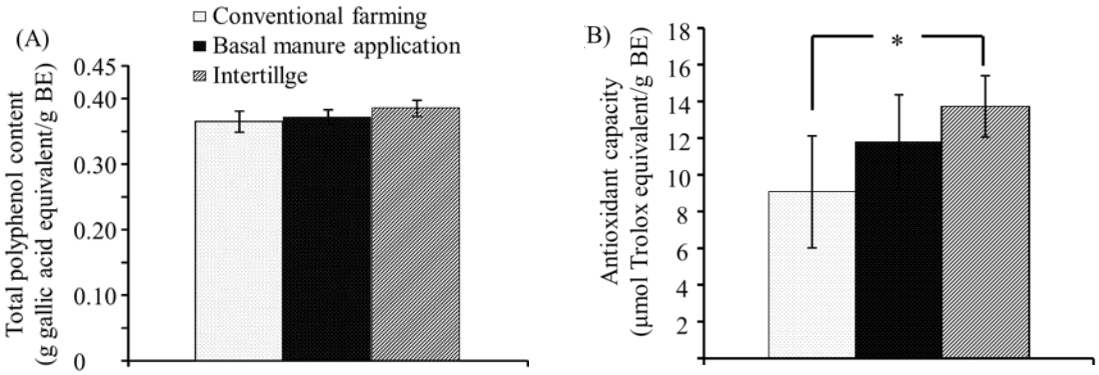


Figure 2.4. Total polyphenol content and antioxidant capacity black soybeans seed coat cultivated by three different fertilization methods. (A) Total polyphenol content is represented as g gallic acid equivalent/g BE. (B) Antioxidant capacity is represented as μmol trolox equivalent/g BE.

2.3.2.2. Flavan-3-ols in plasma of mice given ChronoCare®

A preliminary experiment was conducted to verify whether HPLC-FLD can be applied for the measurements of flavan-3-ols in biological samples and furtherly contribute to the elucidation of the bioavailability of flavan-3-ols. ChronoCare® was orally administrated to mice as a source of mixed flavan-3-ols. The flavan-3-ol composition and the extraction efficiency are shown in Tables 2.3 and 2.4, respectively. After deconjugation, (+)-catechin, (−)-epicatechin,

Table 2.3. Polyphenols content of ChronoCare®

Flavan-3-ols	% (w/w)
(+)-Catechin	0.1
(−)-Epicatechin	5.5
Procyanidin B2	6.5
Procyanidin C1	1.9
Cinnamtannin A2	0.9

Table 2.4. Recovery of flavan-3-ols from plasm

Flavan-3-ols	Recovery (%)
(+)-Catechin	97.3±7
(−)-Epicatechin	129.2±17
Procyanidin B2	96.9±6
Procyanidin C1	71.0±9
Cinnamtannin A2	68.7±4

procyanidin B2, procyanidin C1 and cinnamtannin A2 were detected and quantified as the total amount of corresponding aglycone plus conjugate form. One hour after

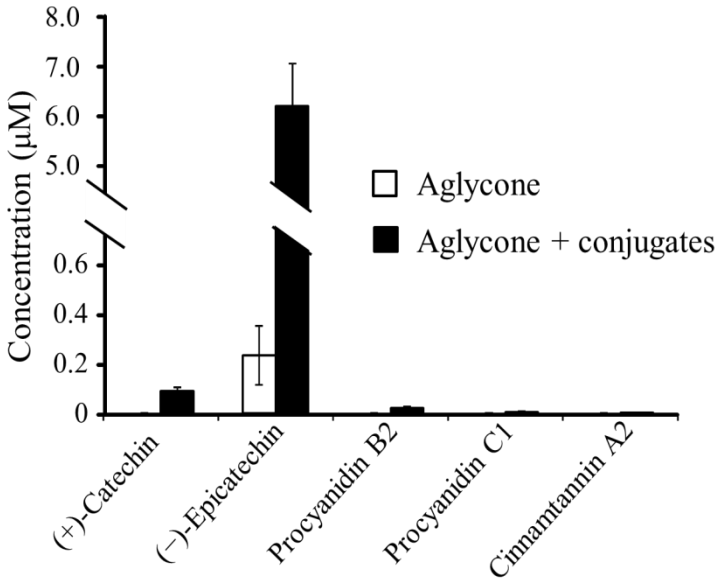


Figure 2.5. Concentrations of flavan-3-ols in mice plasma 1 h after oral administration of BE at a dose of 250 mg/kg body weight.

administration, the concentration of (−)-epicatechin aglycone in plasma was 0.23 μM, and the concentration of its aglycone plus its conjugated forms was 6.21 μM. (+)-Catechin, procyanidin B2, procyanidin C1 and cinnamtannin A2 aglycone were only detected in trace amounts and could not be quantified. The concentration of (+)-catechin aglycone plus its conjugated forms was 0.09 μM, and those of procyanidin B2, procyanidin C1 and cinnamtannin A2 were 0.02, 0.01 and 0.002 μM, respectively (Figure 2.5). (−)-Epicatechin was the only aglycone detected and quantified in the plasma, while (+)-catechin, procyanidin B2, procyanidin C1 and cinnamtannin A2 were detected as broadened peaks, with signals below the LOD.

2.4. Discussion

In this chapter, a new analysis method for black soybean flavan-3-ols using HPLC coupled with a fluorescence detector was developed. Many chromatographic separations used for the determination of flavan-3-ols are based on HPLC-UV. However, their effectiveness was doubted because of the inherent low sensitivity and specificity of UV detection. Using the fluorescence detector, (+)-catechin, (−)-epicatechin, procyanidin B2, procyanidin C1and cinnamtannin A2 were well separated and showed no overlap with any other flavan-3-ol compounds analyzed, including their isomers (Figure 2.1). In

addition, the LOD and LOQ measured using HPLC-FLD was approximately 1000 fold lower than those achieved using HPLC-UV, suggesting the fluorescence detector used in this analysis method optimized detection of flavan-3-ols. The fluorescence properties of flavan-3-ols were first reported by Cho D *et al.* [56], and their peak excitation and emission, were determined to be around 280 nm and 321–324 nm, respectively. These properties have been used to analysis of flavan-3-ols in white wines by Carando S *et al.* [60]. These methods were developed for analyzing flavan-3-ols from food material, but not for analyzing flavan-3-ol in biological samples. The HPLC-FLD developed in this chapter provides the enhanced detection of flavan-3-ols, and it is expected to contribute the investigation of the bioavailability.

Nevertheless, the application of HPLC-FLD to the analysis of flavan-3-ols in food material was explored. Developed HPLC-FLD was used to investigate the flavan-3-ol content in black soybeans cultivated under different fertilization conditions. In recent years, organic farming has increased in popularity. It has been reported that fertilizer type and genotypic differences can influence the phytochemical status of food, and food from organic production may have higher antioxidant capacity from higher polyphenol contents [61-63]. According to the results (Figure 2.3-4), the polyphenol content in black soybean seed coat may yield a higher antioxidant capacity when organic farming methods are used. Further study is required to clarify the cause of the high procyanidin content in BE from soybeans grown with intertillage. This finding was achieved using HPLC-FLD developed in this chapter, suggesting that it can be applied for the measurements of flavan-3-ols in food material.

Finally, a preliminary experiment was conducted to investigate the bioavailability of flavan-3-ols, and results suggested that black soybean flavan-3-ols existed mainly as their conjugated form in the plasma of mice 1 h after oral administration of BE (Figure 2.5). HPLC method applied for the determination of procyanidins in biological samples by using fluorescence detection was first reported by Tanaka N *et al.*, and procyanidin

B2 as well as procyanidin C1 isolated from *Cinnamonomi cortex* were detected in rat plasma [64]. The fluorescence detection was also used by Laurent Y Rios *et al.* and the stability of (+)-catechin, (−)-epicatechin, procyanidin B2, procyanidin C1 in human stomach was evaluated by giving a flavan-3-ol-rich cocoa beverage [65].The recovery of flavan-3-ols in the biological sample was not investigated in neither of the studies, while it was investigated in this chapter to correct the loss of compounds during extraction (Table 2.4), and results showed that flavan-3-ols with higher DP had lower recovery. Considering the recovery of cinnamtannin A2 (68.7%), the 'real' amount of cinnamtannin A2 aglycone and its conjugated forms should be 3.55 nM. However, for further researches that plan to investigate several compounds in various organs, using the recovery to calculated the 'real' amount of flavan-3-ols will waste too much time and effort. Therefore, in the following chapters (Chapter 3 and Chapter 4), calibration curves of corresponding compound in biological samples were used.

In conclusion, a sensitive and specific HPLC method coupled with a fluorescence detector for analysis of flavan-3-ols has been developed in this chapter. This validated analytical method will contribute to the determination of flavan-3-ols in food materials and biological samples for future studies.

Chapter 3. Absorption, metabolism, distribution and faecal excretion of flavan-3-ols in mice after a single oral administration of black soybean seed coat extract

3.1. Introduction

As described in Chapter 1 and 2, flavan-3-ols mainly exist in the seed coat of the black soybeans, and the major compounds are (−)-epicatechin, procyanidin B2, procyanidin C1 and cinnamtannin A2 [25]. *In vivo* experiments demonstrated that black soybean seed coat extract (BE) ameliorated hyperglycaemia and insulin sensitivity through phosphorylation of AMPK [66], and flavan-3-ols isolated from it suppressed acute hyperglycaemia through AMPK phosphorylation and an incretin effect in ICR mice [67].

To achieve beneficial effects *in vivo*, compounds must reach target tissues at effective concentrations. However, pharmacokinetic studies suggested that physiological concentrations of flavan-3-ols were low after their oral consumption, and it is controversial about whether oligomeric flavan-3-ols, namely procyanidins are directly absorbed into the body. It was reported that procyanidin dimers and trimers were detected in the plasma of Wistar rats after oral administration of grape seed procyanidin extract, with procyanidin dimers reaching a concentration of 0.57 nM [49]. In contrast, procyanidin dimers and trimers were not detected in the plasma of rats given purified compounds [68]. These results are conflicting and indicated an inconsistency between the remarkably beneficial effects and low oral bioavailability of flavan-3-ols. Only few studies linked the bioavailability of flavan-3-ols with their *in vivo* beneficial effects. An *in vivo* study of procyanidin dimer B2 suggested that the peak concentration of free procyanidin B2 in the plasma was 0.5 μM and its total urinary excretion within 18 h, including its metabolites, accounted for only about 0.48% of the oral dose [37]. Even with such low oral bioavailability, decreased plasma accumulation of lipid

peroxides was demonstrated in the same study. More detailed research elucidating the bioavailability of flavan-3-ols, as well as measuring their related beneficial effects, are needed.

Therefore, in this chapter, the structurally characterized BE also used in Chapter 2 was orally administrated to ICR mice to investigate the bioavailability of (−)-epicatechin, procyanidin B2, procyanidin C1 and cinnamtannin A2 using developed HPLC-FLD in Chapter 2. Related *in vivo* antioxidant efficiency of BE was also investigated by measuring liver peroxidation.

3.2. Material and methods

3.2.1. Reagents & materials

Same authentic compounds of flavan-3-ols and BE used in Chapter 2 were used. Procyanidin B3-OAc, used as an internal standard, was synthesized and purified by solid phase extraction [69]. HPLC grade methanol, acetonitrile and formic acid were from Wako Pure Chemical Industries (Osaka, Japan). β-Glucuronidase from *E. coli.*, (type IX-A, lyophilized powder), sulfatase from *abalone entrails* (type VIII, lyophilized powder) and 2,6-bis(1,1-dimethylethyl)-4-methylphenol (BHT) were from Sigma-Aldrich Co. LLC (St. Louis, MO, USA). Thiobarbituric acid (TBA) was from Nacalai Tesque Inc. (Kyoto, Japan). 1,1,3,3-Tetramethxypropane (TMP) was from Tokyo Chemistry Industry Co., Ltd. (Tokyo, Japan). All other reagents and chemicals were of analytical grade, unless otherwise stated.

3.2.2. Animal experiments

Animal experiments were approved by the Institutional Animal Care and Use Committee (27-05-09) and performed according to the Guidelines of Animal Experimentation of Kobe University. Male ICR mice (5 weeks old, n = 41) were from

Japan SLC (Shizuoka, Japan) and were maintained at 23 ± 2 °C under an automatic lighting schedule (9:00 am–9:00 pm). The mice were given free access to tap water and commercial chow (Rodent lab diet EQ from Japan SLC) and were acclimatized for two weeks. After acclimatization, ChronoCare® was orally administered at 250 mg/kg body weight after 8 h fasting, with tap water similarly administered to the control group. At 0, 0.25, 0.5, 1, 2, 4, 8 or 24 h after the oral administration, mice were killed by drawing blood by cardiac puncture under anaesthesia with pentobarbital and isoflurane. Plasma was isolated from blood by centrifugation at 3,000 × g for 10 min at 4 °C. Small intestine, liver, kidney, muscle from hind legs, mesenteric fat, epididymal fat and perirenal fat samples were collected and washed with 1.15% KCl. Gastrointestinal content was washed out from the small intestine with1.15% KCl. Faeces samples collected from 5 mice during 0–4, 4–8, 8–12 and 12–24 h time periods was stored at -80 °C until analysis.

3.2.3. Extraction of flavan-3-ols from plasma, tissues and faeces

Tissues and faeces were cut into small pieces and homogenized using a pestle motor mixer. Aliquots of plasma, homogenized tissues and faeces were mixed with 2% (w/v) ascorbic acid to prevent oxidation during extraction and were transferred to perfluoroalkoxy alkanes (PFA) tubes (15 ml, Savillex, Eden Prairie, MN, USA). These plasma and homogenate samples were hydrolysed with β-glucuronidase (500 U/sample) and sulfatase (10 U/sample) for de-conjugation, with incubation for 2 h at 37 °C. To remove lipids, 1 ml hexane was added to each mixture and tubes were vortexed for 30 s and centrifuged at 3000 × g for 10 min. After removal of the hexane layer, 1 ml acetonitrile was added to precipitate the protein and the mixture was again vortexed and centrifuged. Supernatants were collected and each transferred to a new PFA tube and 1 ml ethyl acetate was added to extract the flavan-3-ols. After again vortexing and centrifuging the tubes, the ethyl acetate layers were collected. This extraction step was

repeated two more times. Pooled ethyl acetate layers from each sample were evaporated to dryness. The dried material was dissolved in 50 µl 50% methanol and analysed by a HPLC-FLD. Calibration curves were prepared with plasma or tissues obtained from ICR mice given distilled water spiked with known concentrations of (−)-epicatechin (0–380 µM), procyanidin B2 (0–220 µM), procyanidin C1 (0–40 µM) or cinnamtannin A2 (0–20 µM).

3.2.4. Chromatographic conditions

All quantification of flavan-3-ols was conducted using the HPLC–FLD described in Chapter 2.

3.2.5. Measurement of lipid peroxides in liver with the thiobarbituric acid reactive substances (TBARS) assay

The TBARS assay was performed as described by previous study [70]. Briefly, 250 µl liver homogenate was gently mixed with 50 µl 0.8% (w/v) BHT, 200 µl 8.1% (w/v) sodium dodecyl sulfate (SDS), 1500 µl 20% (v/v, pH 3.5) acetic acetate, 100 µl 36 mM ethylenediaminetetraacetic acid (EDTA) and 1500 µl 0.8% TBA (w/v). The mixture was incubated at 4 °C for 1 h, and then heated in boiling water for 1 h. It was then allowed to cool and 1 ml distilled water and 5 ml n-butanol:pyridine (15:1, v/v) were added. The mixture was then vortexed for 30 s and centrifuged at 2500 × g for 10 min. Absorbance of the upper layer was measured at 532 nm. TMP, ranging from 0–100 µM, was used for calibration curves.

3.2.6. Statistical analyses

Data are expressed as means ± standard deviation. Statistical analysis was performed with Tukey-Kramer multiple comparison test using JMP statistical software version 11.2.0 (SAS Institute, Cary, NC, USA) and the level of significance was $p <$

0.05. Plasma kinetic parameters were calculated using non-compartmental analysis with a program described by Tabata K *et al.* 1999 [71].

3.3. Results

3.3.1. Plasma kinetics of flavan-3-ols

After oral administration of BE to ICR mice at 250 mg/kg body weight, free and conjugated (−)-epicatechin, procyanidin B2, procyanidin C1 and cinnamtannin A2 were detected in the plasma. Kinetic curves are shown in Figure 3.1A and kinetic parameters are shown in Table 3.1. All 4 flavan-3-ols reached maximum concentrations within 1 h and returned to basal levels at 24 h after administration. Free (−)-epicatechin reached a C_{max} of 0.43 μM at 0.80 h. After enzymatic hydrolysis with β-glucuronidase and sulfatase, the concentration, denoted as "Total", was increased and reached a C_{max} of 4.20 μM at 0.38 h. Free procyanidin B2 reached a C_{max} of 0.08 μM, with its total amount reaching a C_{max} of 0.16 μM, at 0.35 h. Free procyanidin C1 reached a C_{max} of 0.04 μM at 0.85 h and its total amount reached a C_{max} of 0.04 μM at 0.35 h. Free cinnamtannin A2 reached a C_{max} of 0.02 μM at 0.40 h and its total amount reached a C_{max} of 0.02 μM at 0.30 h. The C_{max} of total (−)-epicatechin was 10-fold higher than that of free (−)-epicatechin and the C_{max} of total procyanidin B2 was 2-fold higher than that of free procyanidin B2. Similar increases were also observed at other time points. Regarding procyanidin C1 and cinnamtannin A2, the C_{max} values for their total amounts were almost the same as those for their free forms. Compared to the concentrations of free compounds, higher concentrations of total compounds (released by enzymatic hydrolysis) suggested that flavan-3-ols were conjugated *in vivo*. The area under the curve $(AUC)_{(0\rightarrow 24\ h)}$ for total (−)-epicatechin was 6-fold greater than that for free (−)-epicatechin, showing that (−)-epicatechin existed in plasma mainly in its conjugated forms. Not as high as for (−)-epicatechin, the $AUC_{(0\rightarrow 24\ h)}$ values for total procyanidin

Table 3.1. Plasma kinetic parameters for (−)-epicatechin, procyanidin B2, procyanidin C1 and cinnamtannin A2 within 24 h after administration of BE at 250 mg/kg body weight.

Kinetic parameters	(−)-Epicatechin		Procyanidin B2		Procyanidin C1		Cinnamtannin A2	
	Free	Total	Free	Total	Free	Total	Free	Total
C_{max}[a] (μM)	0.43±0.13	4.20±1.60	0.08±0.03	0.16±0.03	0.04±0.01	0.04±0.01	0.02±0.01	0.02±0.01
T_{max}[b] (h)	0.80±0.66	0.38±0.27	0.35±0.12	0.35±0.12	0.85±0.62	0.35±0.12	0.40±0.12	0.30±0.10
$t_{1/2}$[c](h)	5.65±3.81	4.50±2.83	1.28±0.07	1.19±0.21	3.34±0.07	1.12±0.04	3.37±0.01	0.85±0.17
$AUC_{(0\rightarrow 24\ h)}$[d]	1.10±0.32	6.68±1.66	0.13±0.07	0.36±0.21	0.04±0.01	0.13±0.04	0.01±0.01	0.06±0.03
$AUC_{(\infty)}$[e]	1.19±0.43	6.76±1.65	0.15±0.09	0.38±0.25	0.04±0.01	0.13±0.04	0.01±0.01	0.05±0.02
$\%AUC_{extrap}$[f]	5.38±8.01	1.61±1.15	1.88±3.25	4.21±4.75	0.01±0.01	0.01±0.01	0.01±0.01	0.01±0.01

Data are means ± standard deviation.

[a] C_{max} is the maximum plasma concentration.

[b] T_{max} is the time for flavan-3-ols to reach their maximum plasma concentrations.

[c] $t_{1/2}$ is the plasma half-life.

[d] $AUC_{(0\rightarrow 24\ h)}$ is the area under a concentration–time curve from 0 to 24 h, expressed as μmol/L·h.

[e] $AUC_{(\infty)}$ is the area under a concentration–time curve from 0 to infinity, expressed as μmol/L·h.

[f] $\%AUC_{extrap}$ is the area under a concentration–time curve extrapolated from 24 h to infinity, expressed as a percentage of total AUC.

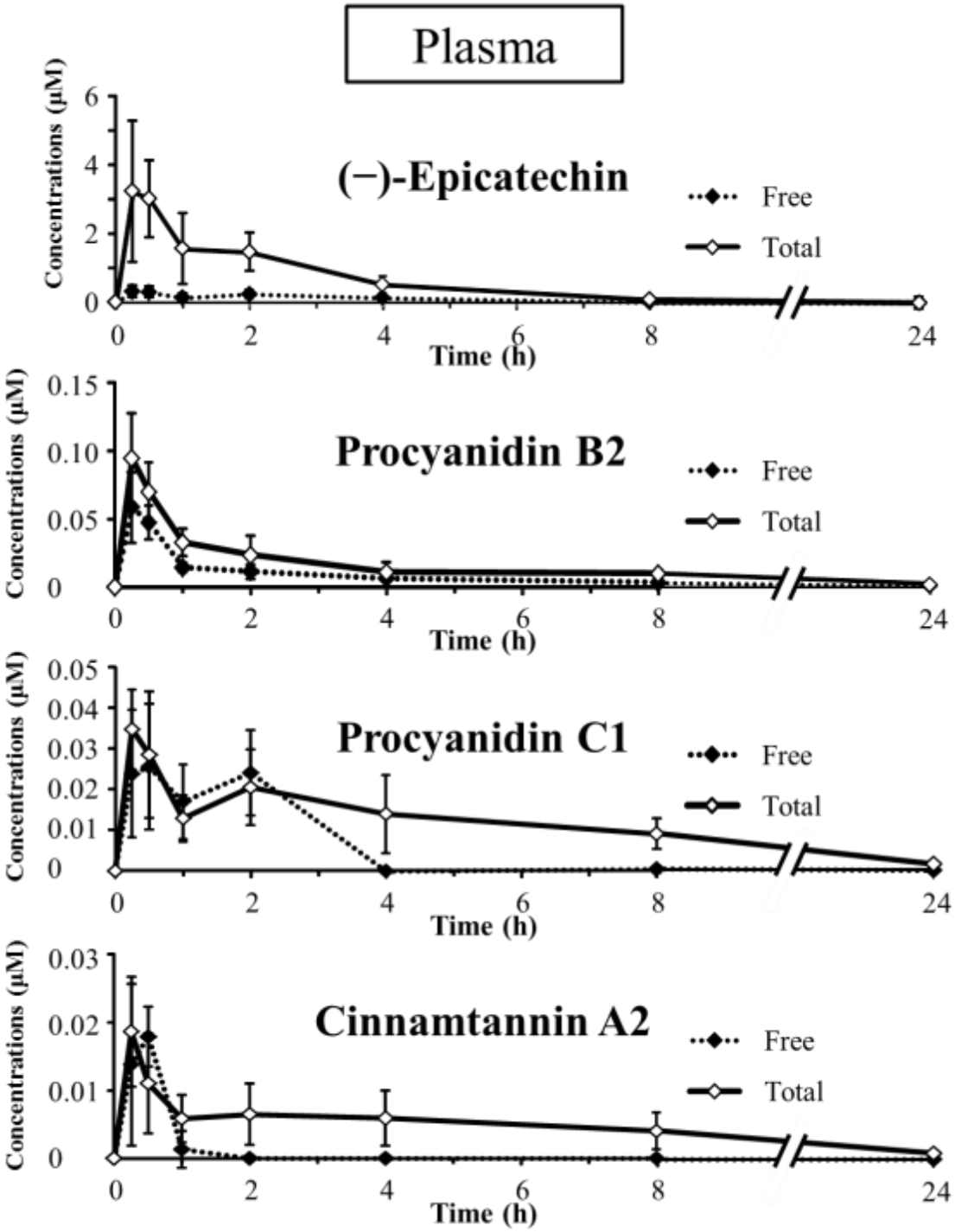


Figure 3.1A. Kinetic curves of flavan-3-ols in the plasma.

B2, procyanidin C1 and cinnamtannin A2 were about 2- to 3-fold greater than those for their corresponding free forms. This indicated that certain amounts of these flavan-3-ols existed in plasma in their free form and some of them also existed in conjugated forms. For all 4 flavan-3-ols, T_{max} values for total compounds were earlier than for free compounds and the $t_{1/2}$ values for total compounds were shorter. This suggested that the conjugates entered the blood stream and were eliminated more quickly than were the free flavan-3-ols. The $\%AUC_{extrap}$ values for all 4 compounds and their conjugates ranged from 0.01%–5.38%, showing that only a small amount of (−)-epicatechin and trace amounts of procyanidin B2, procyanidin C1 and cinnamtannin A2 were left in the plasma after 24 h.

3.3.2. Distribution of flavan-3-ols in tissues

Distribution of free and conjugated (−)-epicatechin, procyanidin B2, procyanidin C1 and cinnamtannin A2 in the small intestine, liver, kidney, muscle, mesenteric fat, epididymal fat and perirenal fat after oral BE administration (Table 3.2, Figure 3.1B-H) were also measured. All 4 flavan-3-ols and their conjugates were found in the small intestine (Figure 3.1B). Consistent with their behaviour in plasma, flavan-3-ols rapidly reached C_{max} levels in the small intestine and only trace amounts were detected at 24 h. The C_{max} values for free (−)-epicatechin, procyanidin B2, procyanidin C1 and cinnamtannin A2 were 90.73, 81.33, 23.94 and 4.48 nmol/g tissue, respectively, and those for the total compounds were 189.86, 183.18, 43.67 and 10.53 nmol/g tissue, respectively. After hydrolysis with β-glucuronidase and sulfatase, the concentrations of the 4 compounds were greatly increased compared with in non-hydrolysed tissues, with these increases observed mainly at 0.25 h. Although slight increases were also observed at other time points, such as with (−)-epicatechin and procyanidin B2 at 0.5 h and cinnamtannin A2 at 1 h, enzymatic hydrolysis did not change the concentration of any flavan-3-ol greatly at most time points. It is noteworthy that approximately half the total

amounts of (−)-epicatechin, procyanidin B2, procyanidin C1 and cinnamtannin A2 were detected at 0.25 h as their free forms. These results indicated that flavan-3-ols were absorbed from the small intestine in both free and conjugated forms and that conjugation seemed to occur only when they were present in this organ.

Only minor increases in liver (−)-epicatechin, procyanidin B2, procyanidin C1 and cinnamtannin A2 were observed after enzymatic hydrolysis (Figure 3.1C), very different results from those in the small intestine. This suggested that, compared with the liver, the small intestine was the major site for conjugation of flavan-3-ols. The C_{max} values for free (−)-epicatechin, procyanidin B2, procyanidin C1 and cinnamtannin A2 in the liver were 1.05, 0.06, 0.04 and 0.06 nmol/g tissue, respectively. The C_{max} values for total (−)-epicatechin, procyanidin B2, procyanidin C1 and cinnamtannin A2 were 1.25, 0.07, 0.04 and 0.05 nmol/g tissue, respectively. Consistent with these results, $AUC_{(0\rightarrow 24\ h)}$ values also showed only minor differences between free and total compounds. This suggested that the flavan-3-ols existed mainly in their free forms or, at least, not as glucuronides and/or sulfates, in the liver.

Similar results were observed in the other tissues analysed in this chapter (Table 3.2, Figure 3.1D–H). Because it included mesenteric lymph nodes, mesenteric fat was the only tissue potentially reflecting flavan-3-ols in the lymphatic system. Concentrations of all flavan-3-ols in mesenteric fat were greatly increased at 0.25–0.5 h after BE administration, with a single sharp peak observed in the kinetic curve over 24 h (Figure 3.1D). The C_{max} values for (−)-epicatechin, procyanidin B2, procyanidin C1 and cinnamtannin A2 were 23.62, 13.82, 4.76 and 2.83 nmol/g tissue, respectively, in the mesenteric fat. These levels were closer to those detected in the small intestine, compared with in other tissues. These results suggested that flavan-3-ols were absorbed by lymphatic transport.

Flavan-3-ols were also detected in the kidney, muscle, epididymal fat and perirenal

fat, mainly in their free forms (Figure 3.1E–H). In the kidney, the C_{max} values for (−)-epicatechin, procyanidin B2 and procyanidin C1 were 0.07, 0.05 and 0.01 nmol/g tissue, respectively. In the muscle, the corresponding C_{max} values reached 0.08, 0.01, 0.01 and 0.005 nmol/g tissue, respectively. In epididymal fat, the C_{max} values for (−)-epicatechin, procyanidin B2 and procyanidin C1 were 0.25, 0.07 and 0.01 nmol/g tissue, respectively. In perirenal fat, the C_{max} values for (−)-epicatechin, procyanidin B2 and procyanidin C1 were 0.99, 0.24 and 0.03 nmol/g tissue, respectively. Among these tissues, (−)-epicatechin, procyanidin B2 and procyanidin C1 showed the highest AUC(0→24 h) values in perirenal fat, and cinnamtannin A2 was detected in only trace amounts in the muscle. Generally, kinetic curves, except those for mesenteric fat, had a tendency to form 2 peaks within 24 h after oral BE administration. The major peaks formed at 0.25–1 h and the minor peaks at 2–4 h.

Table 3.2. $AUC_{(0\rightarrow 24\ h)}$ values for (−)-epicatechin, procyanidin B2, procyanidin C1 and cinnamtannin A2 in various tissues after BE administration at 250 mg/kg body weight.

Tissues	(−)-Epicatechin		Procyanidin B2		Procyanidin C1		Cinnamtannin A2	
	Free	Total	Free	Total	Free	Total	Free	Total
Small intestine	247.23±11.00	310.63±53.07	246.29±13.51	265.14±46.60	79.18±7.75	80.14±7.15	12.24±1.02	12.18±2.44
Liver	2.40±0.33	2.96±0.15	0.87±0.36	0.71±0.01	0.16±0.02	0.26±0.15	0.18±0.02	0.22±0.02
Kidney	0.37±0.21	0.49±0.29	0.12±0.02	0.15±0.02	0.03±0.01	0.06±0.03	N.D.	N.D.
Muscle	0.15±0.03	0.23±0.05	0.05±0.01	0.04±0.01	0.02±0.01	0.04±0.02	0.01±0.00	0.01±0.00
Mesentery	17.47±3.71	11.51±4.89	7.27±6.48	7.43±4.02	2.20±2.28	2.14±1.32	1.18±1.37	0.98±0.82
Epididymal fat	1.77±0.95	1.32±0.03	0.35±0.09	0.32±0.07	0.02±0.01	0.02±0.01	N.D.	N.D.
Perirenal fat	4.80±0.80	5.40±0.87	0.96±0.49	1.44±0.40	0.10±0.12	0.22±0.14	N.D.	N.D.

Data are means ± standard deviation, nmol/g·h. ‘N.D.’, not detected.

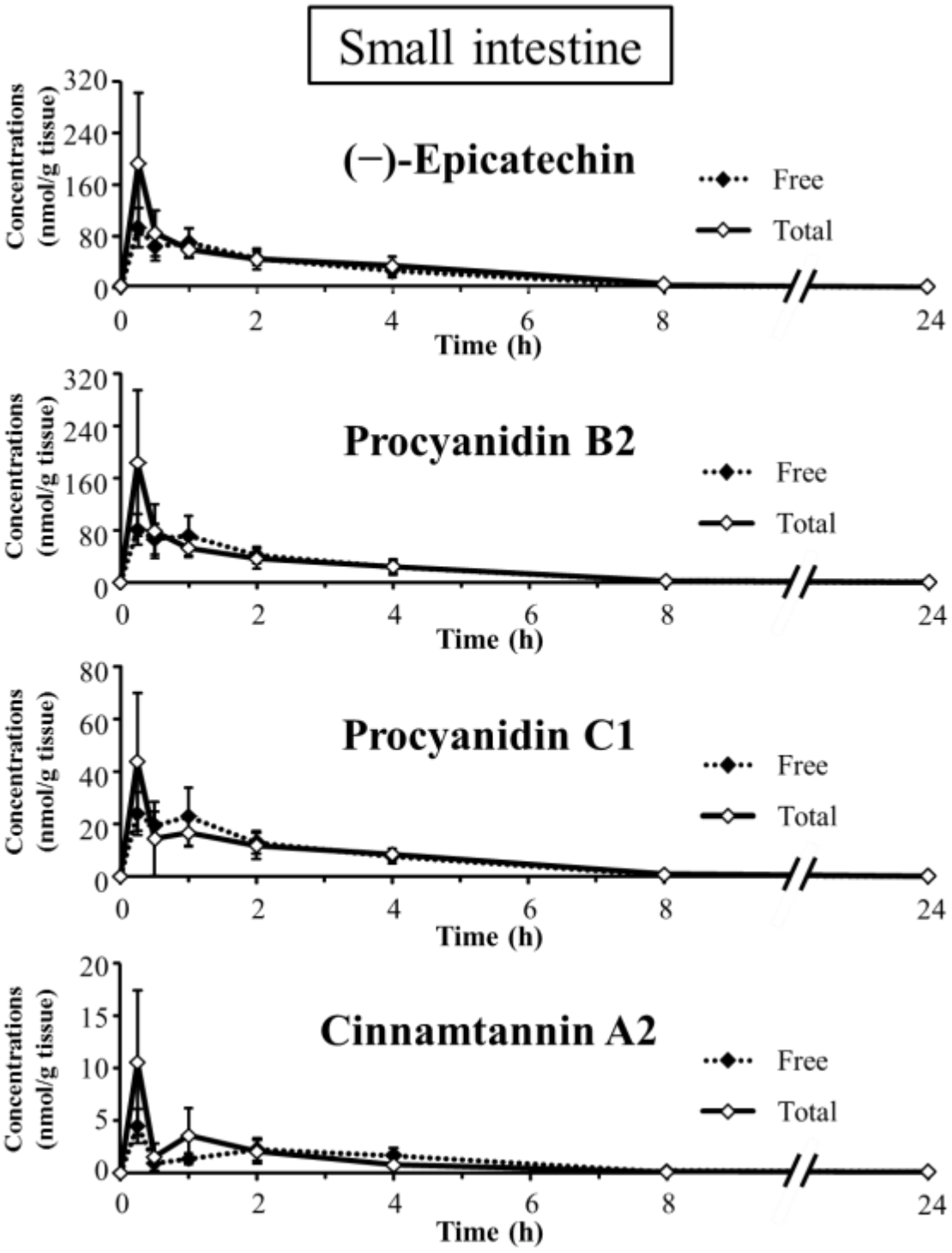


Figure 3.1B. Kinetic curves of flavan-3-ols in the small intestine.

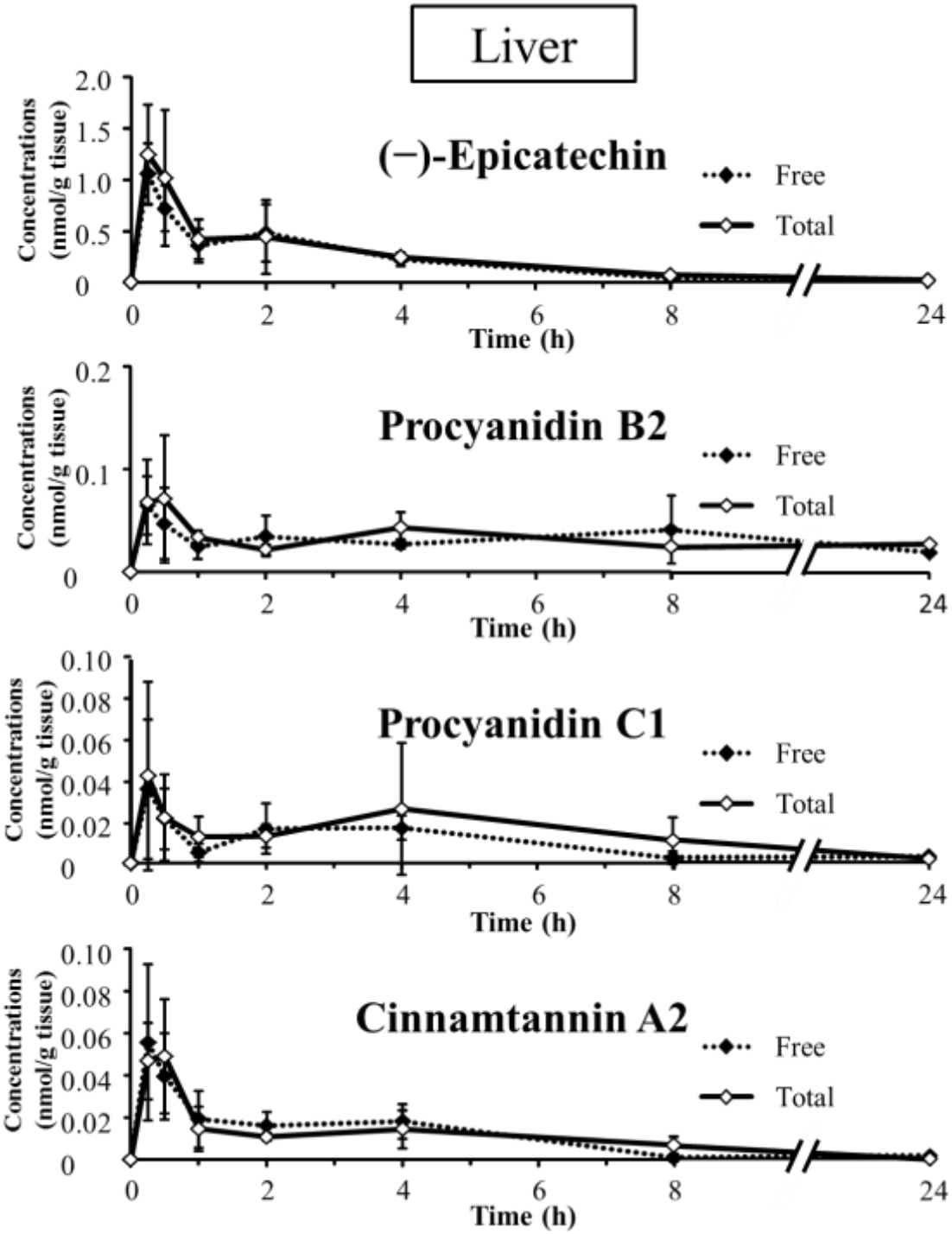


Figure 3.1C. Kinetic curves of flavan-3-ols in the liver.

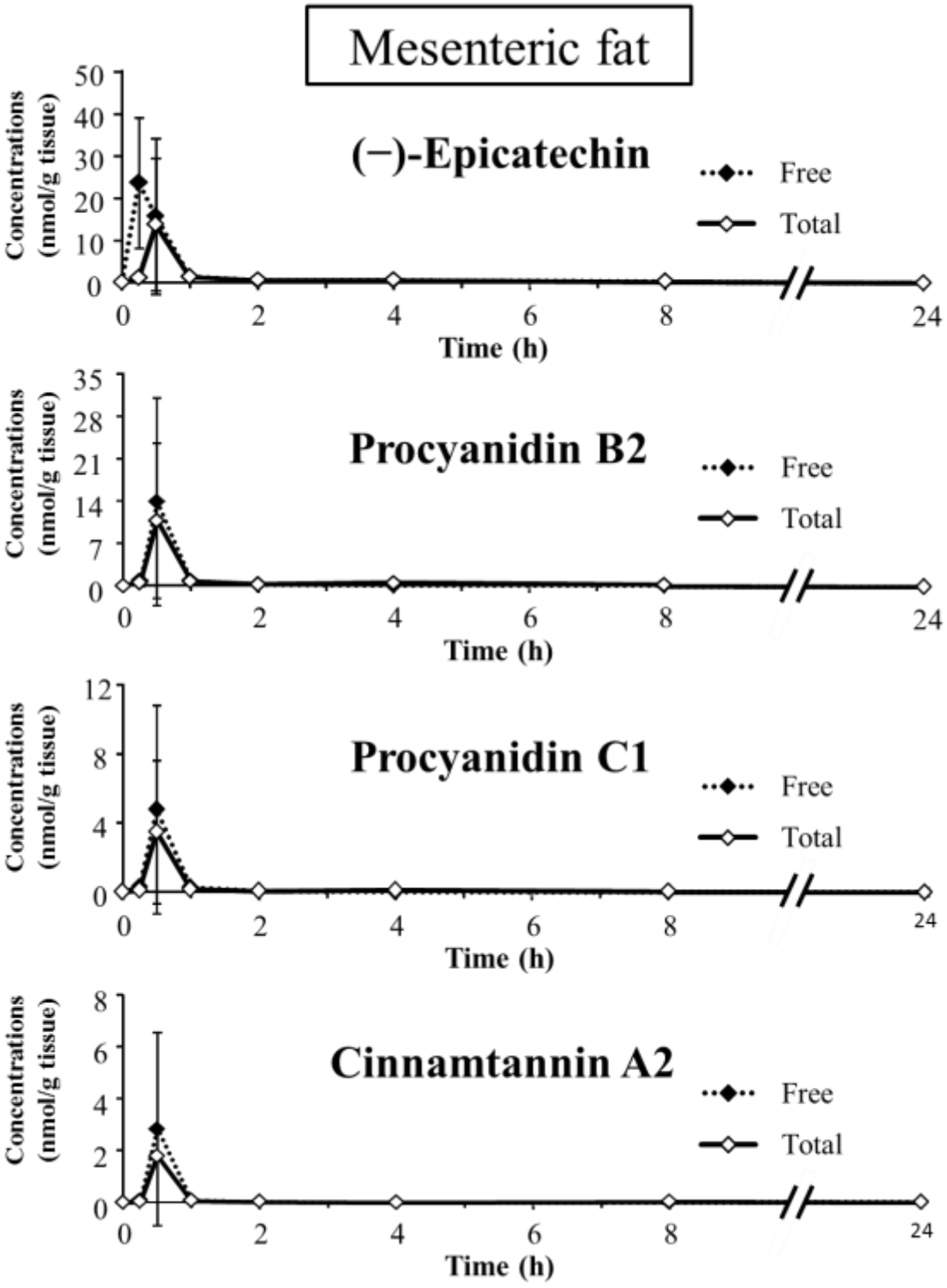


Figure 3.1D. Kinetic curves of flavan-3-ols in the mesenteric fat.

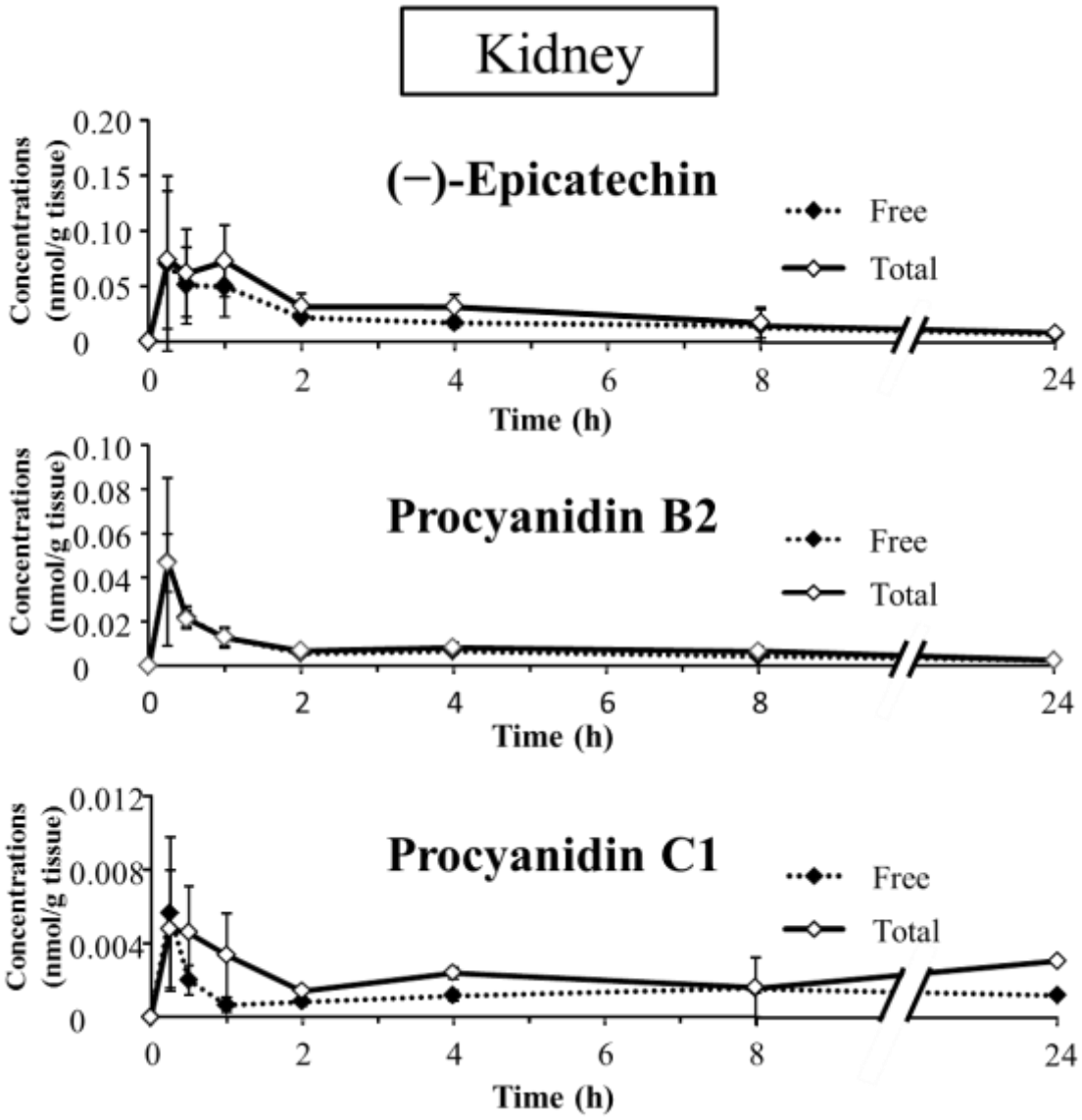


Figure 3.1E. Kinetic curves of flavan-3-ols in the kidney.

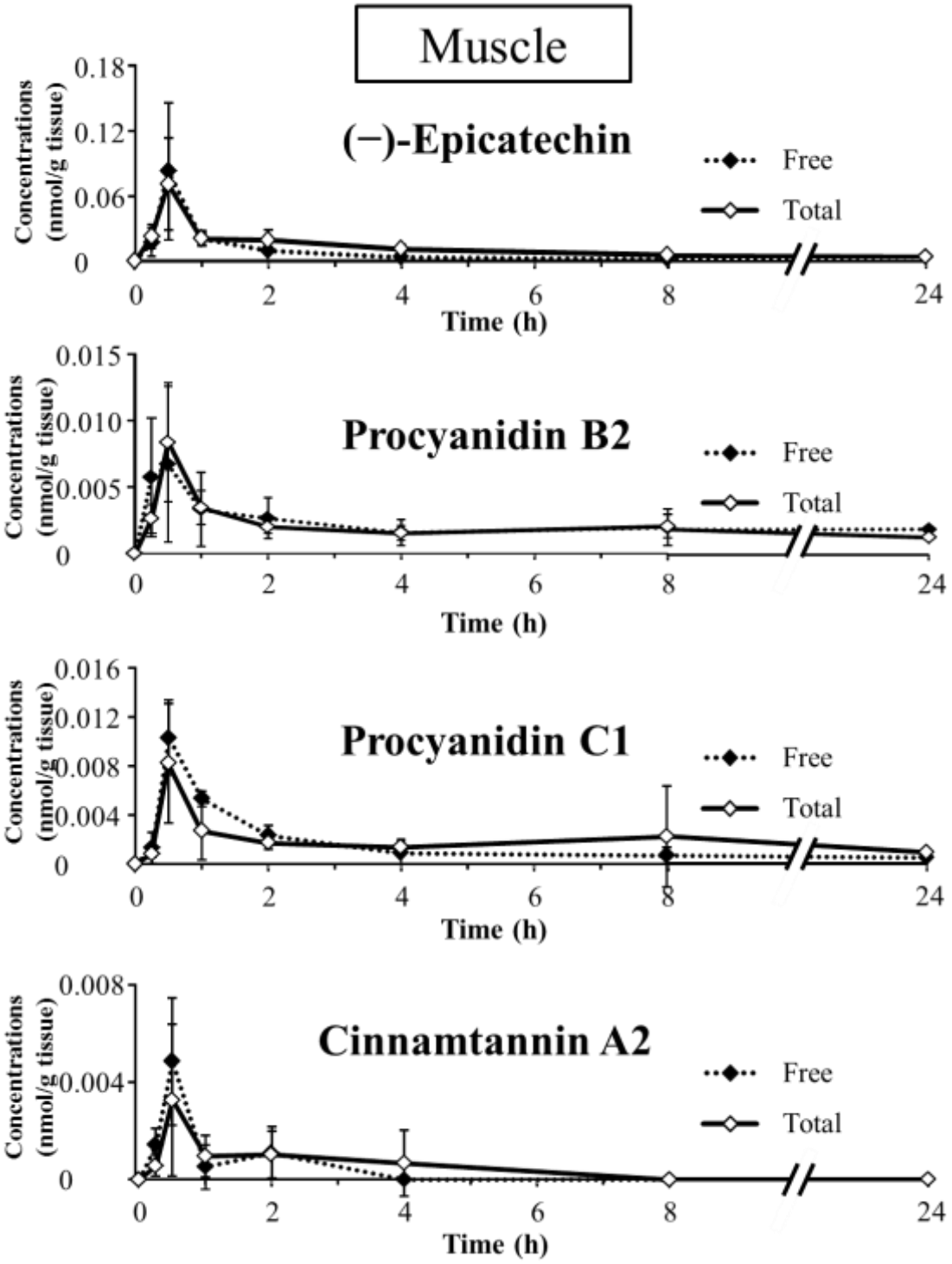


Figure 3.1F. Kinetic curves of flavan-3-ols in the muscle.

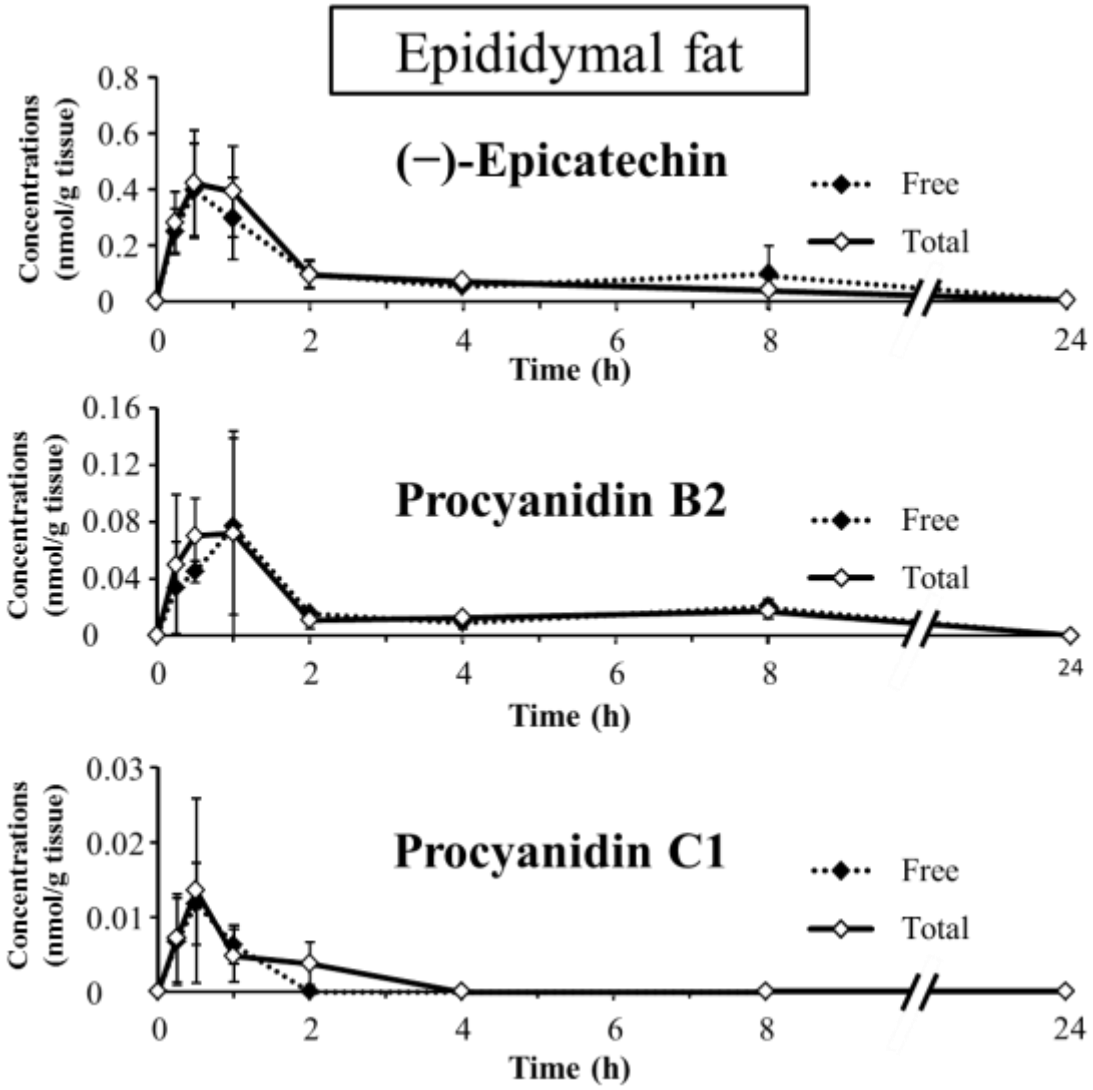


Figure 3.1G. Kinetic curves of flavan-3-ols in the epididymal fat.

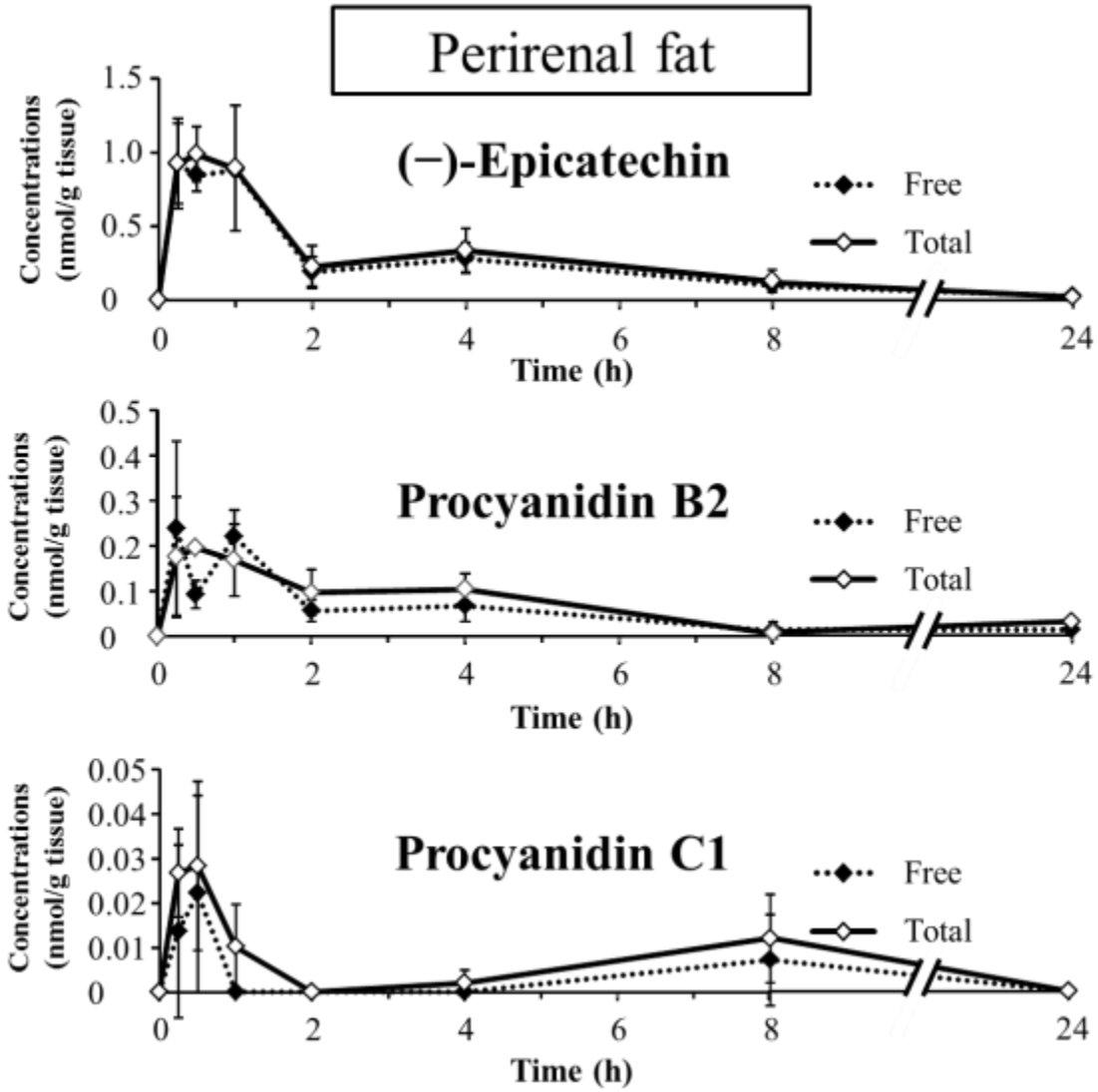


Figure 3.1G. Kinetic curves of flavan-3-ols in the perirenal fat.

3.3.3. Faecal excretion of flavan-3-ols

Faeces were collected every 4 h during the first 12 h after oral BE administration and, otherwise, during 12–24 h. For comparisons, concentrations of flavan-3-ols were

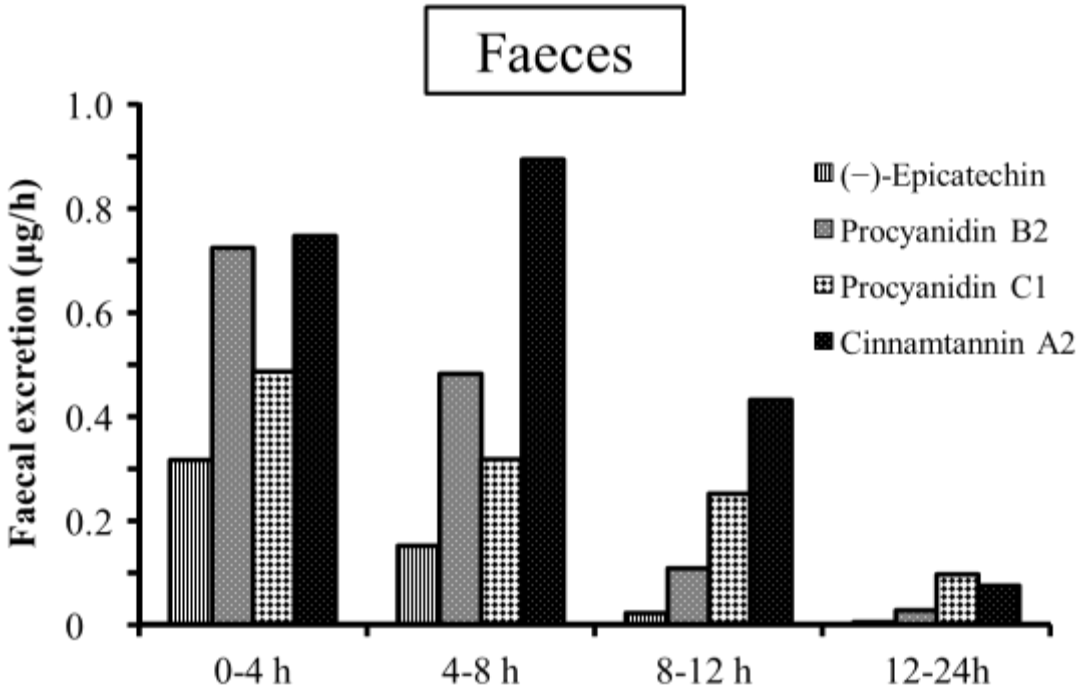


Figure 3.2. Excreted amounts of flavan-3-ols in mouse faeces after oral administration of BE at 250 mg/kg body weight. Absolute amounts of flavan-3-ols are expressed as μg/h/mouse.

represented as μg/h (Figure 3.2). (−)-epicatechin, procyanidin B2 and procyanidin C1 were excreted to faeces at the highest levels from 0–4 h, at 0.32, 0.73 and 0.49 μg/h, respectively. Excretion of cinnamtannin A2 was increased once from 0–8 h, reaching maximum excretion (0.89 μg/h) from 4–8 h. It was then decreased from 8–12 h. Within 24 h, 0.45% of (−)-epicatechin, 1.06% of procyanidin B2, 3.45% of procyanidin C1 and 12.30% of cinnamtannin A2 were excreted to faeces after oral BE administration.

3.3.4. Antioxidant effects of BE on lipid peroxidation in the liver

As shown in Figure 3.3, significantly decreased, by approximately 38%,

malonyldialdehyde (MDA) levels were observed at 2 h after BE administration, compared with in 0 h liver samples. Decreasing trends were also observed at 0.25, 0.5 and 1 h, but without statistical significance. After 2 h, MDA levels returned to basal values (0 h level) at 4 h and then were slightly decreased again at 8 and 24 h. This 2-peak pattern is similar to the kinetic curves of flavan-3-ols, suggesting that this *in vivo* antioxidant efficiency was closely related to the flavan-3-ols detected in the liver after BE administration.

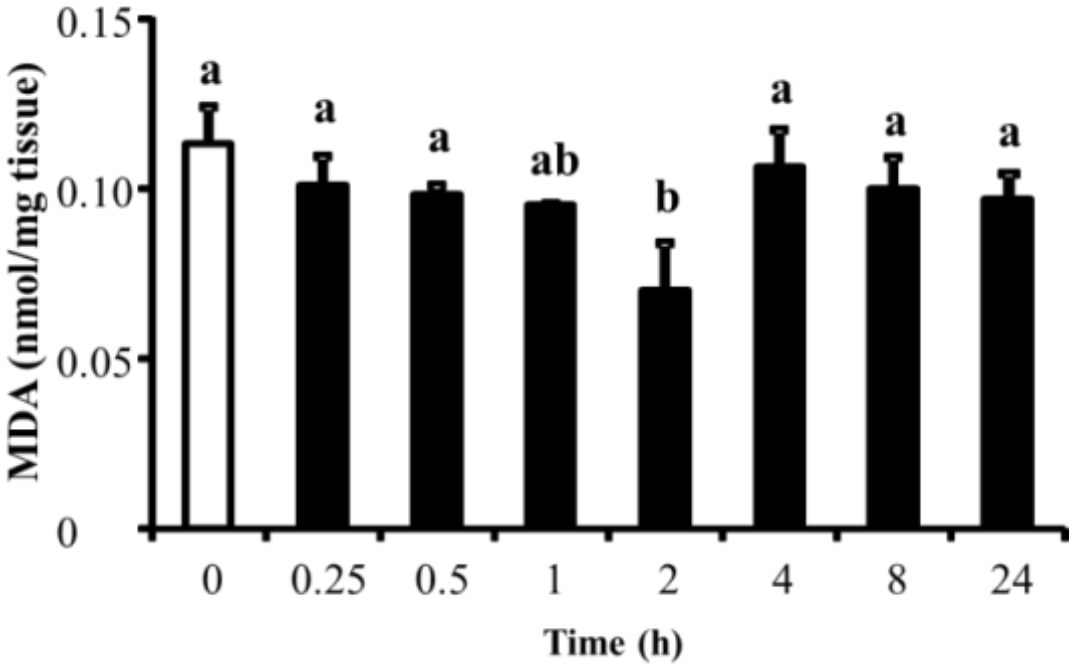


Figure 3.3 Lipid peroxidation products in the liver after oral administration of BE at 250 mg/kg body weight. Lipid peroxidation products were measured with the TBARS assay and are represented as the amount of MDA. Results are means ± standard deviation. Superscripted letters indicate significant differences among groups, determined by the Tukey–Kramer multiple comparison test (p <0.05).

3.4. Discussion

In this chapter, a structurally characterized flavan-3-ol-rich extract, BE was used to evaluate bioavailabilities of (−)-epicatechin, procyanidin B2, procyanidin C1 and cinnamtannin A2. Although it would be better to analyze bioavailability with pure compounds instead of food or plant extracts, extraction and purification of each

flavan-3-ol, except monomers, from food or plants is very difficult. Therefore, food material or their extracts containing high amount of flavan-3-ols have often been used for bioavailability studies [49, 72, 73]. However, most of the extracts used previously were not structurally well characterized. Moreover, the previous reports did not quantify each flavan-3-ol and presented sums of compounds with the same degrees of polymerization, such as monomers, dimers, trimers and tetramers. Besides their degrees of polymerization, based on linkages between flavan-3-ol units, procyanidins are also classified into A- and B-type procyanidins, and a few studies suggested that these two types had different bioavailabilities and bioactivities [74, 75]. Therefore, it is important to use a structurally characterized flavan-3-ol-rich extract. In this chapter, a commercially available BE consisting of 5.5% (−)-epicatechin, 6.5% procyanidin B2, 1.9% procyanidin C1 and 0.9% cinnamtannin A2 was orally administered to ICR mice at 250 mg/kg. This dose was equivalent to 13.8 mg/kg (−)-epicatechin, 16.3 mg/kg procyanidin B2, 4.8 mg/kg procyanidin C1 and 2.4 mg/kg cinnamtannin A2. In addition, as described in Chapter 2, analytical method greatly affects the limit of determination of flavan-3-ols in biological samples. These limitations may cause underestimating the bioavailability of polyphenols. Therefore, quantification of polyphenols in this dissertation was conducted using calibration curves prepared with plasma or tissues obtained from ICR mice given distilled water. These calibration curves minimized the matrix effect of biological samples on the quantification (Table 3.3).

Table 3.3. Calibration curves of flavan-3-ols in biological samples

Biological samples	(−)-Epicatechin		Procyanidin B2		Procyanidin C1		Cinnamtannin A2	
	Equation	R^2	Equation	R^2	Equation	R^2	Equation	R^2
Plasma	y=0.6274x	0.9958	y=0.4294x	0.9867	y=0.2181x	0.9743	y=0.0555x	0.9914
Small intestine	y=0.4898x	1.0000	y=0.3402x	0.9999	y=0.2406x	0.9990	y=0.1214x	0.9994
Liver	y=0.8422x	0.9943	y=0.4119x	0.9713	y=0.2048x	0.9197	y=0.0907x	0.9084
Mesenteric fat	y=0.5944x	1.0000	y=0.4939x	0.9995	y=0.3902x	0.9970	y=0.1765x	0.9987
Kidney	y=52.095x	0.9604	y=11.821x	0.8965	y=5.7577x	0.8919	y=1.7000x	0.9248
Muscle	y=23.335x	0.9860	y=12.811x	0.9788	y=4.7232x	0.9783	y=0.8865x	0.9685
Epididymal fat	y=0.6323x	0.9986	y=0.4391x	0.9970	y=0.1693x	0.9992	y=0.0230x	0.9728
Perirenal Fat	y=0.6303x	0.9999	y=0.4625x	0.9995	y=0.2119x	0.9963	y=0.0774x	0.9841

y is represented as the peak area ratio of flavan-3-ol and internal standard, and x is represented as concentration, μM.

Before entering the blood stream, orally administered BE first reached the gastrointestinal tract. It was reported that flavan-3-ols are stable in the stomach [65]. Results in this chapter indicated that BE reached the small intestine and its constituent flavan-3-ols were metabolized to conjugates during absorption. This conjugation mainly occurred at the early stage of their residence in the small intestine (Figure 3.1B). Results also demonstrated that some of the flavan-3-ols were absorbed into the body from the small intestine and distributed to the tissues in their free forms.

In the plasma, most (−)-epicatechin existed as its conjugated forms, with a small portion in its free form. Small amounts of procyanidin B2, procyanidin C1 and cinnamtannin A2 were also detected (Figure 3.1A, Table 3.1). As to (−)-epicatechin, opposite results were reported: Some studies reported that both free and conjugated (−)-epicatechin were detected in the plasma [76, 77], while others reported that no free (−)-epicatechin, only conjugated (−)-epicatechin was detected [78, 79]. Results of the former studies indicated that (−)-epicatechin were absorbed through 2 ways: one is through efflux in their free form; and the other is through conjugation in their conjugated forms. Results in this chapter were consistent with the former studies suggesting (−)-epicatechin were absorbed from small intestine, where conjugation mainly occurred. As to procyanidins, they were once considered not absorbable because of their high molecule weights, such as procyanidin B2 and procyanidin C1 [68]. More attention was, thus, devoted to potential degradation of procyanidins into smaller units or their metabolism into phenolic acids by microflora [80-82]. Results in this chapter showed that procyanidin B2, procyanidin C1 and cinnamtannin A2 were also absorbed in 2 ways, but the conjugation of them was not as obvious as that of (−)-epicatechin (Figure 3.1A, Table 1). Similar results were reported by other studies [49, 74, 83]: Shoji *et al.* reported that, after enzymatic hydrolysis, procyanidin concentrations in the plasma were slightly higher than that of non-hydrolysed plasma of rats after orally administration of apple procyanidins. Xiao *et al.* identified 14 conjugates in rats after

oral administration of pure procyanidin B2 [84]. Direct absorption of procyanidin trimers and tetramers into the plasma was reported, but there is no report on the bioavailability of procyanidin C1 and cinnamtannin A2. Thus, findings in this chapter are the first to report the existence and distribution of procyanidin C1 and cinnamtannin A2 *in vivo*.

Liver is the principal site of phase II metabolism. However, unlike in the small intestine, only slight conjugation of flavan-3-ols was observed in the liver (Figure 3.1C). This suggested that flavan-3-ols are conjugated mainly in the small intestine rather than in the liver. Dose was reported to affect metabolism of flavan-3-ols [83]: When a flavan-3-ol-rich extract was orally administered to rats at a high dose (2 g/kg), metabolism appeared to occur mainly in the liver, while at a lower dose (1 g/kg), metabolism was primarily in the small intestine. Although this theory was not proved, the dose of BE in this dissertation (250 mg/kg body weight) was much lower than these studies. Notably, the flavan-3-ols were conjugated primarily in the small intestine in both studies, even though different extracts were used.

Results with mesenteric fat may have indicated that flavan-3-ols were absorbed, at least in part, through lymphatic transport (Figure 3.1D). Quercetin, a flavonoid with a similar molecular weight to that of (−)-epicatechin, was found in lymph fluid as well as in the plasma after intraduodenal administration [85]. In contrast, glucuronide and methylated forms of (−)-epicatechin were the primary compounds that detected in lymphoid tissues of rats fed a cocoa-rich diet for 3 weeks [86]. Molecules which are highly lipophilic or with high molecular weight can enter the systemic circulation through the intestinal lymphatics to avoid liver metabolism [87, 88]. Results in this chapter clearly demonstrated that relatively high concentrations of free flavan-3-ols were detected in the mesenteric fat, compared to that in other tissues 0.25–0.5 h after oral administration of BE. These results agreed with the theory that a portion of the flavan-3-ols, upon reaching the small intestine, could be absorbed from the intestinal

lymphatics without conjugation and then appear in the plasma and tissues without being metabolized. Detection of flavan-3-ol metabolites in lymphoid tissues in another study [86] was potentially attributable to the long experimental period, because absorption through the intestinal lymphatics seemed to occur soon after administration.

Rather than the urinary, faecal excretion of flavan-3-ols was investigated in this chapter. Because metabolic conversion of flavan-3-ols caused by microflora was not specifically determined, the total amount of flavan-3-ols absorbed into the body, including free compounds, conjugates and/or metabolites generated by microflora, can be evaluated by measuring faecal excretion. Extremely low faecal excretion of (−)-epicatechin was observed in this chapter, which is coincide with other studies [76, 79, 89], indicating that (−)-epicatechin had the highest bioavailability, followed by procyanidin B2, procyanidin C1 and cinnamtannin A2. A study using ^{14}C labelled-compound showed that approximately 40% of orally administered procyanidin B2 was excreted into faeces within 24 h [90]. This excretion amount is much higher than the excretion (1.06%) observed in our study. One potential explanation is the ^{14}C labelling method can estimate not only free and conjugated compound but also its degradation products, such as phenolic acids produced by microflora. Tsang *et al.* reported that only trace amounts of free procyanidin dimers and trimers were detected in faeces of rats after oral administration of a grapeseed extract, but tetramers were not analysed [91]. Results in this chapter showed that molecular weight and/or DP of flavan-3-ols not only had a major impact on the amounts excreted in faeces but also affected the excretion rates (Figure 3.2).

Significant inhibition of lipid peroxidation in the liver 2 h after administration of BE was observed (Figure 3.3). The suppressed pattern of lipid oxidation was consistent with appearance of free (−)-epicatechin, procyanidin B2, procyanidin C1 and cinnamtannin A2 in the liver (Figure 3.1C). *In vivo* antioxidative efficiency after a single administration of flavan-3-ols or flavan-3-ol-rich food extracts were reported.

Koga *et al.* reported that copper ion-induced lipid oxidation was remarkably suppressed in the plasma 1 h after oral administration of proanthocyanidin-rich grapeseed extract to rats [92]. However, they identified only gallic acid, (+)-catechin, and (−)-epicatechin but not procyanidins in the plasma. Others reported that a single dose of pure procyanidin B2 decreased plasma accumulation of lipid peroxides in the liver of rats after treatment with a free radical generator and copper ions [37]. In the same study, approximately 0.4 μM free procyanidin B2 was detected. Based on all these results, incorporated flavan-3-ols may cause direct antioxidative efficiency *in vivo*.

In conclusion, this chapter reported the bioavailability of (−)-epicatechin, procyanidin B2, procyanidin C1 and cinnamtannin A2 after the intake of BE. These results contribute to the further understanding of flavan-3-ols as the functional ingredient in black soybeans, and predict the possible bioavailability of flavan-3-ols in future human research. Moreover, observed beneficial effects in vivo and corresponding physiological concentrations of polyphenols discussed in this chapter are very important for studies about function of food.

Chapter 4. Black soybean improves vascular function and oxidative stress associating with polyphenol concentration in the body of healthy human

4.1. Introduction

In this chapter, to investigate that whether consumption of black soybean can exert health beneficial effects to human, an 8-week intervention trial was conducted to explore the effect of black soybean on the vascular function and oxidative stress in healthy human.

Vascular function is important to the pathogenesis of the cardiovascular diseases (CVD) [93, 94]. Studies suggest that vascular dysfunction due to aging and vascular stiffness is associated with an increased risk of CVD [95, 96]. In addition, injurious stimuli such as oxidative stress and inflammatory mediators result in the dysfunction of vascular endothelial, primarily through a reduction in the nitric oxide (NO) may also trigger the onset of CVD [97].

Emerging evidence supports that the dietary intake of polyphenols, particularly flavonoids, might be able to exert some beneficial vascular effects and reduce the risk of CVD [98]. Previous study performed on ovariectomized rats indicated that 10-week consumption of black soybean (35% of experiment diet) inhibited oxidative stress by increasing antioxidant activity and improving lipid profiles, and the risk factors associated with CVD were greatly improved [99]. Another study performed on rats indicated that oral administration of 50 and 100 mg/kg body weigh black soybean extract for 14 days reduced the risk of CVD by improving blood circulation through inhibition of platelet aggregation and thrombus formation [26]. For human trial, Lee M. *et al*. reported that black soybean testa extract supplementation (2.5 g/day) for 8 weeks improved visceral fat and plasma lipid profiles in overweight Korean adults [39]. No human trial has been reported on the beneficial effects of black soybean consumption on reducing the risk of CVD yet. Moreover, although polyphenols were considered

responsible for these effects, physiological concentrations of corresponding polyphenols after the consumption of test materials, and the correlation between the observed effects and polyphenol concentrations were not investigated or discussed in previous studies. Therefore, more detailed human studies are needed.

4.2. Methods and reagents

4.2.1. Design of human study

This study was approved and conducted by the Institutional Review Board of Fujicco Co., Ltd. (Trial registration: 5702), and it was performed in accordance with the Declaration of Helsinki, and written informed consent was obtained from all volunteers. Volunteers were excluded if they fulfilled any of the following criteria: (1) have a history of severe gastrointestinal disease, liver disease, kidney disease or heart disease; (2) currently undergoing treatment for metabolic syndrome or its associated diseases; (3) using medication for blood or blood pressure, such as Warfarin and Captopril; (4) whose physical condition were considered inappropriate for this study by doctors. The main inclusion criterion was that the vascular age of volunteer was higher than her chronological age. Forty-seven female volunteers aged 20 to 70 were finally enrolled for this study. Volunteers were asked to consume 30 g/day of roasted black soybeans for 8 weeks without strict restrictions on the intake patterns. Test materials were from Fujicco Co., Ltd (Kobe, Japan), composition of which was shown in Table 4. During the 8-week trial, volunteers were asked to return to the facility at the 4^{th} and the 8^{th} week for the measurements of anthropometrics, accelerated plethysmogram (APG), blood pressure and assessments of health-related quality of life (HRQOL), as well as blood and urine collection under fasting condition before breakfast. These measurements and collection were also conducted before the trial began (0 week).

Table 4. Composition of roasted black soybean

Nutrition component (per 30 g)	
Calories	132 kcal
Protein	10.6 g
Fat	6.5 g
Carbohydrate	5.0 g
Dietary fiber	5.6 g
Sodium	1.8 mg
NaCl	0.0 g
Calcium	50.7 mg
Polyphenol content (per 30 g)	
Anthocyanidin	
Cyanidin-3-*O*-glucoside	12.4 mg
Flavan-3-ols	
(−)-Epicatechin	0.3 mg
Procyanidin B2	0.5 mg
Procyanidin C1	1.2 mg
Cinnamtannin A2	1.6 mg
Isoflavones	
Daizein	0.5 mg
Daidzin	78.1 mg
Glycitein	0.1 mg
Glycitin	0.5 mg
Genistein	1.1 mg
Genistin	48.4 mg

4.2.2. Measurements of anthropometrics

Body weight, BMI, body fat percentage, visceral fat percentage, biological age, basal metabolic rate, estimated bone mass and muscle mass was measured using body composition meter (BC-610-PB, TANITA. Co., Ltd. Tokyo, Japan).

4.2.3. Measurements of vascular function

Vascular function was measured using APG by a “Pulse Analyzer” device (Pulse Analyzer Plus View, YKC Corporation, Tokyo, Japan). APG was classified into *a*, *b*, *c* and *d* waves, namely, early systolic positive wave, early systolic negative wave, late

systolic re-increasing wave and late systolic re-decreasing wave, respectively. Their magnitudes and the height ratios of *b/a*, *c/a* and *d/a* were measured. In addition, vascular age, vascular waveform, waveform score and peripheral vascular health were also measured using the Pulse Analyzer. Systolic and diastolic blood pressure was measured in the right upper arm using automated sphygmomanometer (HEM-9000AI, OMRON Corporation, Kyoto, Japan).

4.2.4. Assessments of HRQOL

HRQOL was assessed using the Japanese version of the 36-item Short-Form Health Survey (SF-36) questionnaire developed by iHope International Co., Ltd. It consists of 36 questions categorized into 8 domains, including physical functioning, social functioning, physical role, emotional role, bodily pain, vitality, metal health and general health. The 8 domains were furtherly aggregated into 3 summary measures: physical component summary score, metal component summary score and role/social component summary score. The score on each of the eight health concepts ranges from 0 to 100, and a higher score correspond to better HRQOL [100].

4.2.5. Measurements of biomarkers in blood and urine

Blood and urine were collected from each volunteer under fasting condition before breakfast. Plasma isolated from the blood and urine was used for the measurements of nitric oxide (NO), 8-hydroxy-2'-deoxyguanosine (8-OHdG), hexanoyl-lysine (HEL) and myeloperoxidase (MPO) by using corresponding commercial kit respectively [NO: NO2/NO3 Assay Kit-C II (Colorimetric)~Griess Reagent Kit~ (DOJINDO LABORATORIES, Kumamoto, Japan); 8-OHdG: New 8-OHdG Check ELISA (Japan Institute for the Control of Aging, NIKKEN SEIL Co., Ltd (JaICA) Sizuoka, Japan); HEL: HEL ELISA kits ((JaICA), Sizuoka, Japan); MPO: Human serum MPO and urine MPO ELISA kits ((JaICA) Sizuoka, Japan)]. Urinary NO was calculated by creatinine

equivalent. Urinary creatinine levels were measured by CREATININE ASSAY KIT COLORIMETRIC for URINE SAMPLE 96 tests (Cayman CHEMICAL, Michigan, USA).

In addition, hematologic parameters including creatinine, total protein (TP), blood urea nitrogen (BUN), estimated glomerular filtrationrate (eGFR), glucose (GLU), lactate dehydrogenase (LDH), alkaline phosphatase (ALP), γ- glutamyltranspeptidase (γ-GTP), creatine kinase (CK), aspartate aminotransferase (AST), alanine aminotransferase (ALT), triglycerides (TG), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), Na, Cl, K, white blood cells (WBC), red blood cells (RBC), hemoglobin (Hb), hematocrit (Ht), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean hemoglobin concentration (MCHC) and platelet (PLT) were analyzed by LSI Medience Corporation (Tokyo, Japan).

4.2.6. Extraction of polyphenols from the plasma and urine

An aliquot of 10 ml urine was concentrated to 2 ml before analysis. Concentrated 2 ml of urine or 500 μl of plasma were mixed with 2% (w/v) ascorbic acid to prevent oxidation during extraction and were transferred to polypropylene centrifuge tubes (15 ml, BD Bioscience, NJ, USA) which were siliconized using Sigmacote® (Sigma, MO, USA). These plasma and urine samples were hydrolyzed with β-glucuronidase (500 U/sample) and sulfatase (10 U/sample) for de-conjugation, with incubation for 2 h at 37 °C. Solid phase extraction (SPE) was used to extract polyphenols from the mixture. C18 Sep-Pak cartridges (1cc, 50 mg, Waters, MA, USA) were conditioned with 5 ml of methanol and 5 ml of Milli-Q water. Samples were centrifuged at 3000 × g for 15 min to precipitate the protein for loading. Cartridges were then washed with 5 ml of 10% methanol and 2 ml of 95% methanol was added to elute the polyphenols. The polyphenol fraction from each sample was evaporated to dryness and finally dissolved

in 50 µl 50% methanol for analysis using HPLC.

4.2.7. Chromatographic conditions

HPLC was performed using a system equipped with a DGU-20A 3R degassing unit, LC-20AD XR binary pump, SIL-20AC XR auto sampler, RF-20A XS fluorescence detector, SPD-M20A diode array detector, CTO-20AC column oven and CBM-20A communications bus module connected to an LC work station (Shimadzu Corporation, Kyoto, Japan). The analytical column was a Cadenza CL-C18 column (φ 250 mm × 4.6 mm, 3 µm, Imtakt, Kyoto, Japan), protected by a guard column (Cadenza CL-C18, φ 5 mm × 2 mm, 3 µm, Imtak, Kyoto, Japan). All quantification of polyphenols was conducted using the HPLC system as described previously [69] with modifications.

For the analysis of cyanidin-3-*O*-glucoside, 10% (v/v) formic acid was mobile phase A and formic acid:acetonitrile (10:90, v/v) was mobile phase B. Elution of cyanidin-3-*O*-glucoside was achieved using these linear gradients: 15% B over 0–10 min; 80% B over 10-25 min; and 15% B, over 25-40 min. The elution from 25–40 min re-equilibrated the column between samples. The flow rate was 0.8 ml/min, the injection volume was 10 µl and the temperature of the column oven was 40 °C. Absorbance of cyanidin-3-*O*-glucoside was measured with wavelength of 513 nm.

For the analysis of flavan-3-ols and isoflavones, 0.1% (v/v) formic acid was mobile phase A and acetonitrile was mobile phase B. Separation was achieved using these linear gradients: 5%–10% B over 0–5 min; 10%–85% B over 5-65 min; 30-45% B, over 65-110 min; 80% B, over 110–120 min; and 5% B over 120-140 min. The elution from 120-140 min re-equilibrated the column between samples. The flow rate was 0.7 ml/min, the injection volume was 10 µl and the temperature of the column oven was 40 °C. Fluorescence of the flavan-3-ols was measured with excitation and emission wavelengths of 276 and 316 nm, respectively. Absorbance of isoflavones was measured with wavelengths of 254 nm and 210 nm.

4.2.8. Reagents & materials

Cyandin-3-*O*-glucoside, daidzein, daidzin and genistein were from Wako Pure Chemical Industries (Osaka, Japan), glycitein, glycitin and genistin were from Extrasynthese (Genay, France), and *S*-equol was from Cayman Chemicals (MI, USA). Flavone from Wako Pure Chemical Industries (Osaka, Japan) was used as an internal standard for isoflavones. HPLC grade methanol, acetonitrile and formic acid were from Wako Pure Chemical Industries (Osaka, Japan). β-glucuronidase from *E. coli.,* (type IX-A, lyophilized powder) and sulfatase from *abalone entrails* (type VIII, lyophilized powder) were from Sigma-Aldrich Co. LLC (St. Louis, MO, USA).

4.2.9. Statistical analysis

Data are expressed as means ± standard deviation. Statistical analysis was performed with Dunnett`s test using JMP statistical software version 11.2.0 (SAS Institute, Cary, NC, USA) and the level of significance was $p<0.05$. Pearson correlation coefficient was applied to determine the association between physiological concentrations of polyphenols and corresponding factors, including anthropometric measurements, biomarkers of vascular function and oxidative stress, and hematologic parameters. Statistical analysis of correlation efficient was performed using *t*-tests, and the level of significance was $p<0.05$.

4.3. Results

4.3.1. General characteristics of volunteers

Before the trial began, the average vascular age of volunteers was 39.2±10.2 years old with an average BMI of 20.8±2.8 kg/m^2. The vascular age was significantly higher than the chronological age which was 33.5±10.0 years old ($p<0.01$). At the end of the trial, 3 volunteers dropped out for non-health related reasons and 44 volunteers

completed the 8-week trial with no report on poor health or any abnormality. No significant changes in anthropometric and hematologic parameters were observed throughout the trial (Table 4.1, Table 4.2).

Table 4.1. Anthropometric parameters of 44 volunteers during the 8-week trial.

Anthropometric parameters	0 week	4th week	8th week
Body weight (kg)	52.6±1.03	52.71±1.04	52.78±1.02
BMI	20.82±0.42	20.87±0.42	20.89±0.42
Body fat (%)	26.80±0.82	27.46±0.79	27.64±0.82
Visceral fat (%)	3.45±0.30	3.50±0.31	3.55±0.31
Biological age	27.93±1.66	29.02±1.67	29.32±1.72
Basal metabolic rate (kcal/day)	1140±15	1134±14	1133±14
Estimated bone mass (kg)	2.20±0.04	2.17±0.04	2.17±0.04
Muscle mass (%)	36.03±0.42	35.78±0.43	35.50±0.49

Table 4.2. Hematologic parameters of 44 volunteers during the 8-week trial.

Hematologic parameters	0 week	4th week	8th week
TP	7.37±0.35	7.38±0.31	7.33±0.32
BUN	11.35±2.88	12.98±3.07	12.04±2.42
CRE	0.69±0.08	0.67±0.08	0.66±0.07
GLU	88.21±5.86	89.74±6.01	86.63±6.36
LDH	164.74±19.34	165.37±21.28	163.04±21.10
ALP	157.49±38.93	164.72±42.78	165.84±45.81
γ-GTP	17.05±11.03	18.26±11.94	17.88±13.20
AST	17.63±5.37	19.02±4.29	18.28±5.05
ALT	13.53±6.87	14.60±6.21	14.28±6.49
TG	61.37±34.32	63.28±35.00	60.30±29.58
LDL-C	102.72±30.02	101.51±28.71	101.02±29.03
HDL-C	68.74±11.41	68.60±13.47	71.16±12.48
Na	138.53±1.69	137.65±1.40	138.21±1.42
Cl	104.60±1.59	103.63±1.49	103.23±1.91
K	4.32±0.29	4.39±0.24	4.48±0.32
WBC	5582±1633	5441±1477	5698±1457
RBC	440±29	452±26	449±27
Hb	13.04±0.91	13.19±0.87	13.28±0.98
Ht	39.78±2.16	40.59±2.06	41.06±2.42
MCV	90.77±4.65	90.59±4.55	91.63±4.63
MCH	29.72±1.95	29.21±1.82	19.64±1.92
MCHC	32.75±0.93	32.47±0.80	32.31±0.82
PLT	25.21±6.24	25.93±7.10	25.89±6.21

4.3.2. Improved vascular function

During 8-week trial, vascular function was significantly improved (Table 4.3). The

Table 4.3. Effect of 8-week consumption of roasted black soybean on vascular function by measuring accelerated plethysmography

Vascular function	**0 week**	**4th week**	**8th week**
APG			
Vascular age	39.27±10.2	37.36±9.69*	37.07±9.61*
a wave	107.07±9.27	107.39±11.8	110.80±9.15*
b wave	−59.07±14.6	−65.32±15.9*	−67.98±13.9*
c wave	−23.48±10.8	−18.52±10.6*	−17.89±11.1*
d wave	−35.43±13.4	−29.91±12.2*	−30.02±13.5*
b/a	−0.55±0.13	−0.61±0.14*	−0.61±0.11*
c/a	−0.22±0.10	−0.17±0.10*	−0.16±0.10*
d/a	−0.33±0.13	−0.28±0.12*	−0.27±0.12*
Vascular waveform	2.84±1.26	2.43±0.94*	2.27±0.84*
Waveform score	53.34±11.9	58.41±10.8*	59.95±10.0*
Peripheral vascular health	64.93±12.8	70.59±10.1*	72.41±8.69*
Blood pressure			
Systolic blood pressure	112.09±14.4	109.59±14.0	111.73±10.9
Diastolic blood pressure	67.66±10.9	66.30±11.5	67.39±8.32

Means ± SE are shown. * $p < 0.05$ *vs.* 0 week, *Dunnett`s* Test.

vascular age became about 2 years lower at the end of the trial compared to that at the 0 week. In detail, 25 volunteers showed lowered vascular age at the 4th week, and it increased to 26 volunteers at the 8th week. In total, 33 volunteers showed lowered vascular age during the trial. As to the rest 11 volunteers, vascular age of 8 volunteers did not change throughout the trial, and that of 3 volunteers was slightly higher at the end of the trial compared to that at the 0 week. Indicators of APG that reflect the vascular function were significantly improved: *a*, *c*, *d* wave magnitude and the height ratios *of c/a* and *d/a* were increased; *b* wave magnitude and height ratio of *b/a* were decreased. The significant change was observed at both 4th and 8th week. In addition, significantly improved vascular waveform, waveform score and peripheral vascular health were also observed. Systolic and diastolic blood pressure decreased at the 4th

and/or the 8th week, but significant change was not observed.

Based on the timing of lowered vascular age being observed, results of 25 volunteers whose lowered vascular age was observed at the 4th week were extracted and denoted as 'Improved at the 4th week' group; results of 26 volunteers whose lowered vascular age was observed at the 8th week were extracted and denoted as 'Improved at the 8th week' group in this study. Further results of 3 groups, including 'Total' group (results of 44 volunteers), 'Improved at the 4th week' group and 'Improved at the 8th week' group, were shown.

4.3.3. Increased NO concentration

NO is a central factor in the regulation of vascular function, including blood pressure and blood flow [101]. In this chapter, the amount of NO in the urine and plasma was investigated. NO in the urine of 'Total' group increased significantly at the 4th and 8th week compared to that of 0 week (Figure 4.1). Significantly increased NO was also observed in the urine of 'Improved at the 4th week' group and 'Improved at the 8th week' group more clearly compared to that of the 'Total' group. NO in the plasma slightly increased, but no significant change was observed in either group during the trial.

4.3.4. Improved oxidative stress

Representative biomarkers of oxidative stress including 8-OHdG, HEL and MPO were investigated. 8-OHdG in the plasma of 'Total' group decreased at the 4th and 8th week, and significant change was observed at the 8th week (Figure 4.2A). Significant decreased 8-OHdG was also observed at the 4th after results being stratified and furtherly analyzed. In the urine, greatly decreased 8-OHdG was also observed, but none of them was significant. Decreased HEL in the plasma was observed with significant change at the 8th week in all 3 groups, and no significant change was observed in the

urine (Figure 4.2B). Decreased MPO was observed in the plasma and the urine of all groups, and it decreased greatly in the urine at the 8th week (Figure 4.2C).

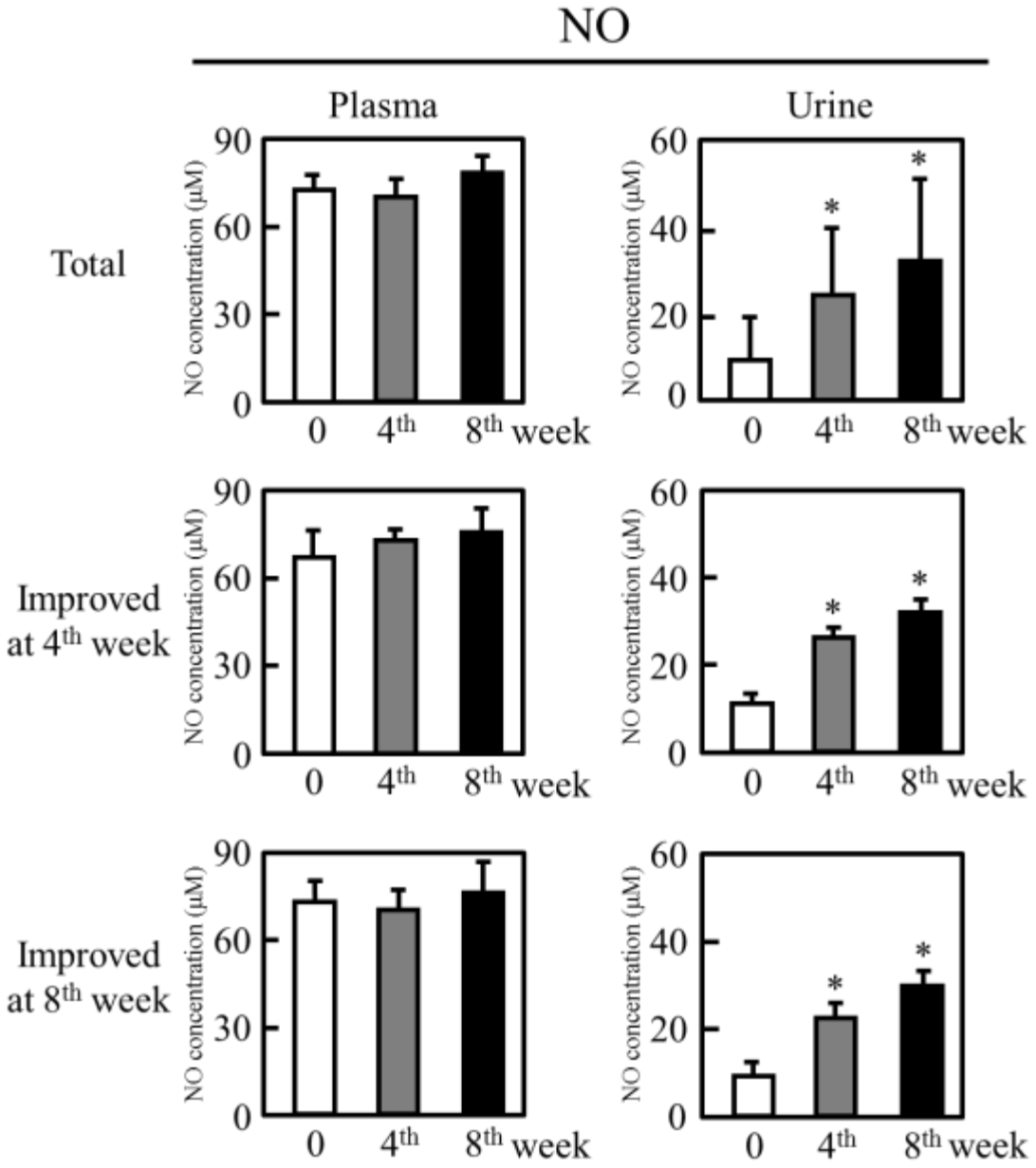


Figure 4.1. The effect of black soybean consumption on NO concentrations in the plasma and urine. NO was measured using corresponding kit and represented as means ± standard deviation. *$p < 0.05$ *vs.* 0 week, *Dunnett`s* Test. Results of 44 volunteers were denoted as 'Total', and results of volunteers who showed reversed vascular age at the 4th or 8th week were denoted as 'Improved at the 4th week' and 'Improved at the 8th week', respectively.

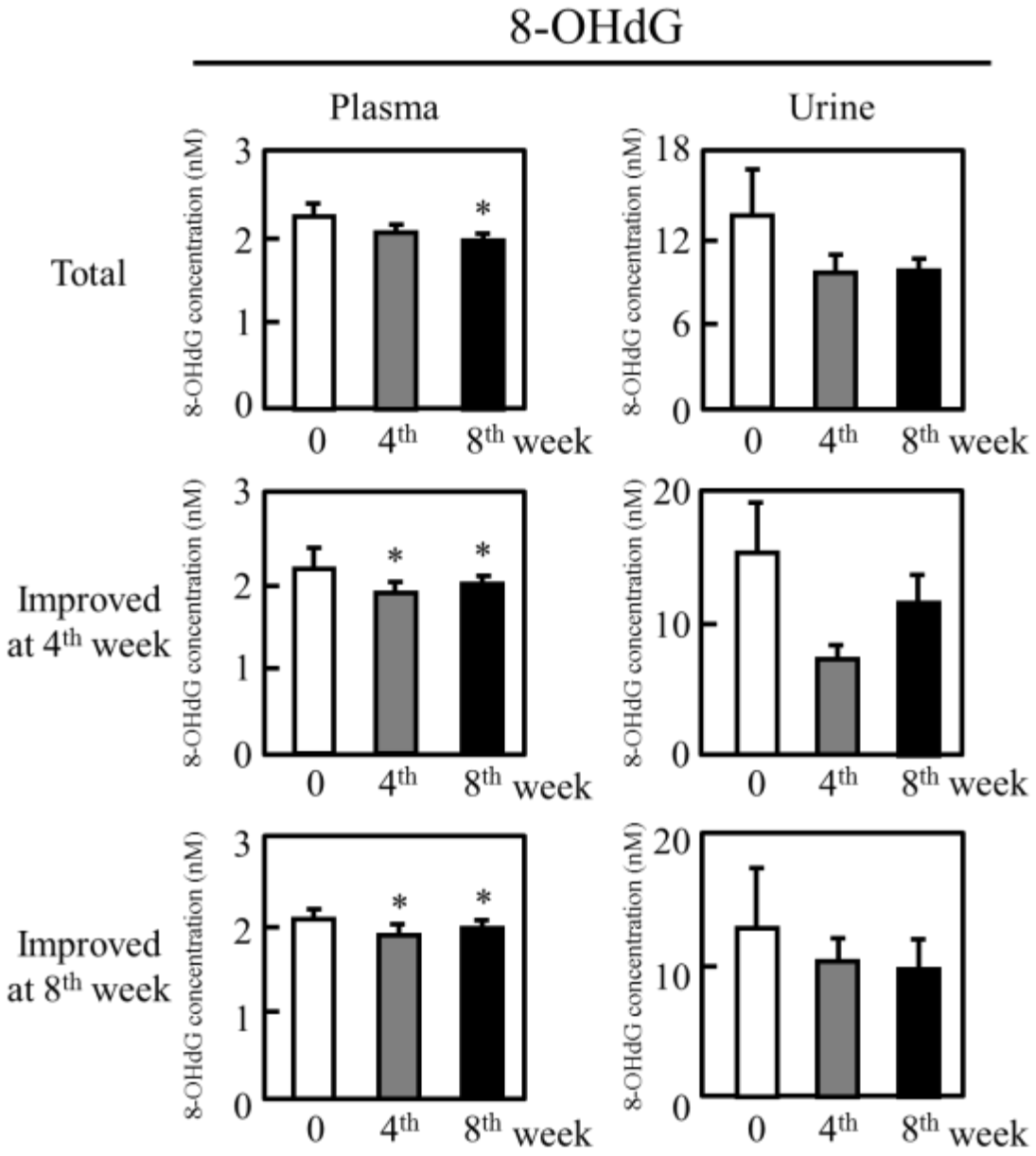


Figure 4.2A. The effect of black soybean consumption on biomarkers related to8-OHdG concentrations in the plasma and urine. 8-OHdG was measured using corresponding kit and represented as means ± standard deviation. *$p < 0.05$ *vs.* 0 week, *Dunnett`s* Test. Results of 44 volunteers were denoted as 'Total', and results of volunteers who showed reversed vascular age at the 4th or 8th week were denoted as 'Improved at the 4th week' and 'Improved at the 8th week', respectively.

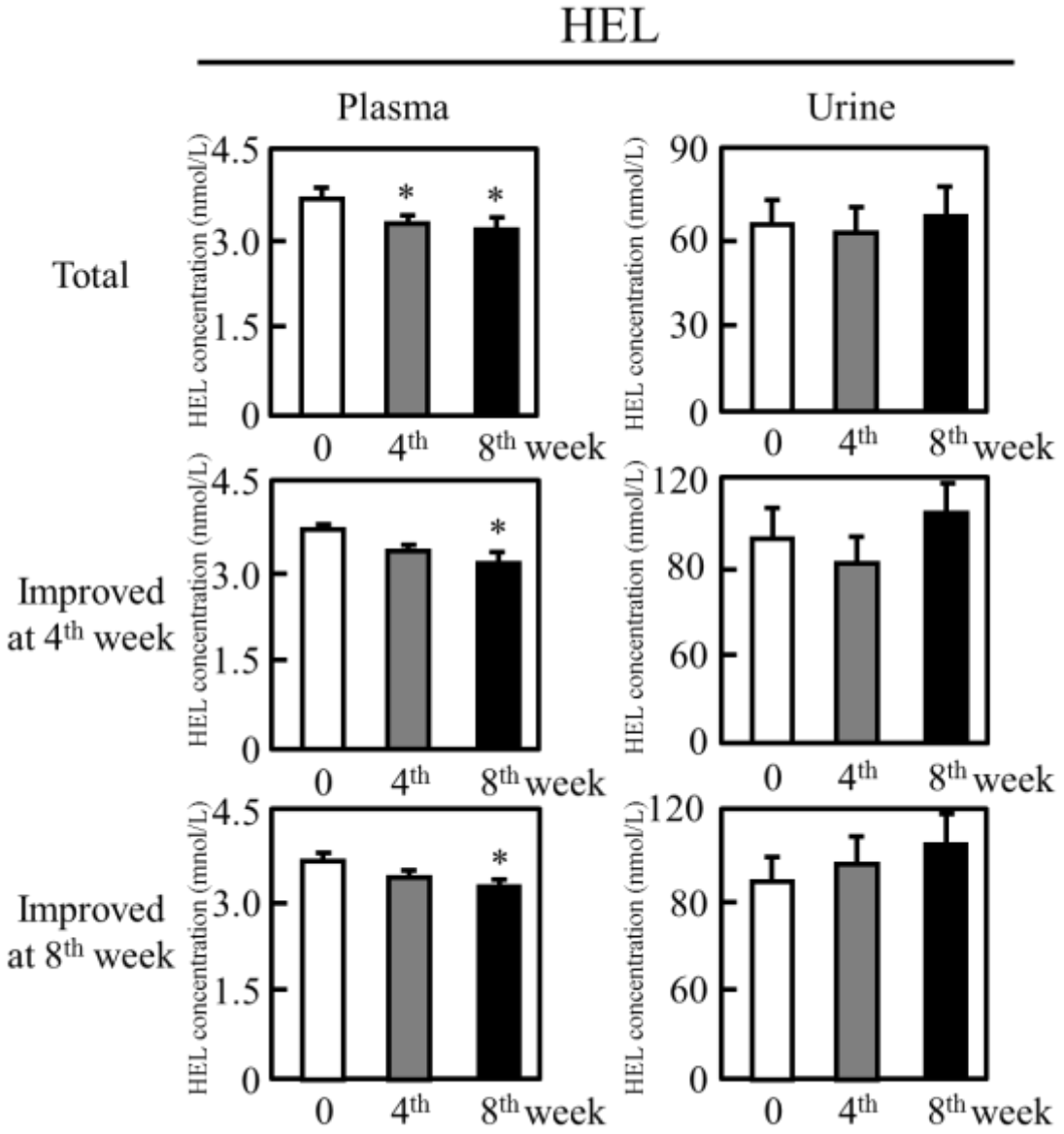


Figure 4.2B. The effect of black soybean consumption on biomarkers related toHEL concentrations in the plasma and urine. HEL was measured using corresponding kit and represented as means ± standard deviation. *$p < 0.05$ *vs.* 0 week, *Dunnett`s* Test. Results of 44 volunteers were denoted as 'Total', and results of volunteers who showed reversed vascular age at the 4th or 8th week were denoted as 'Improved at the 4th week' and 'Improved at the 8th week', respectively.

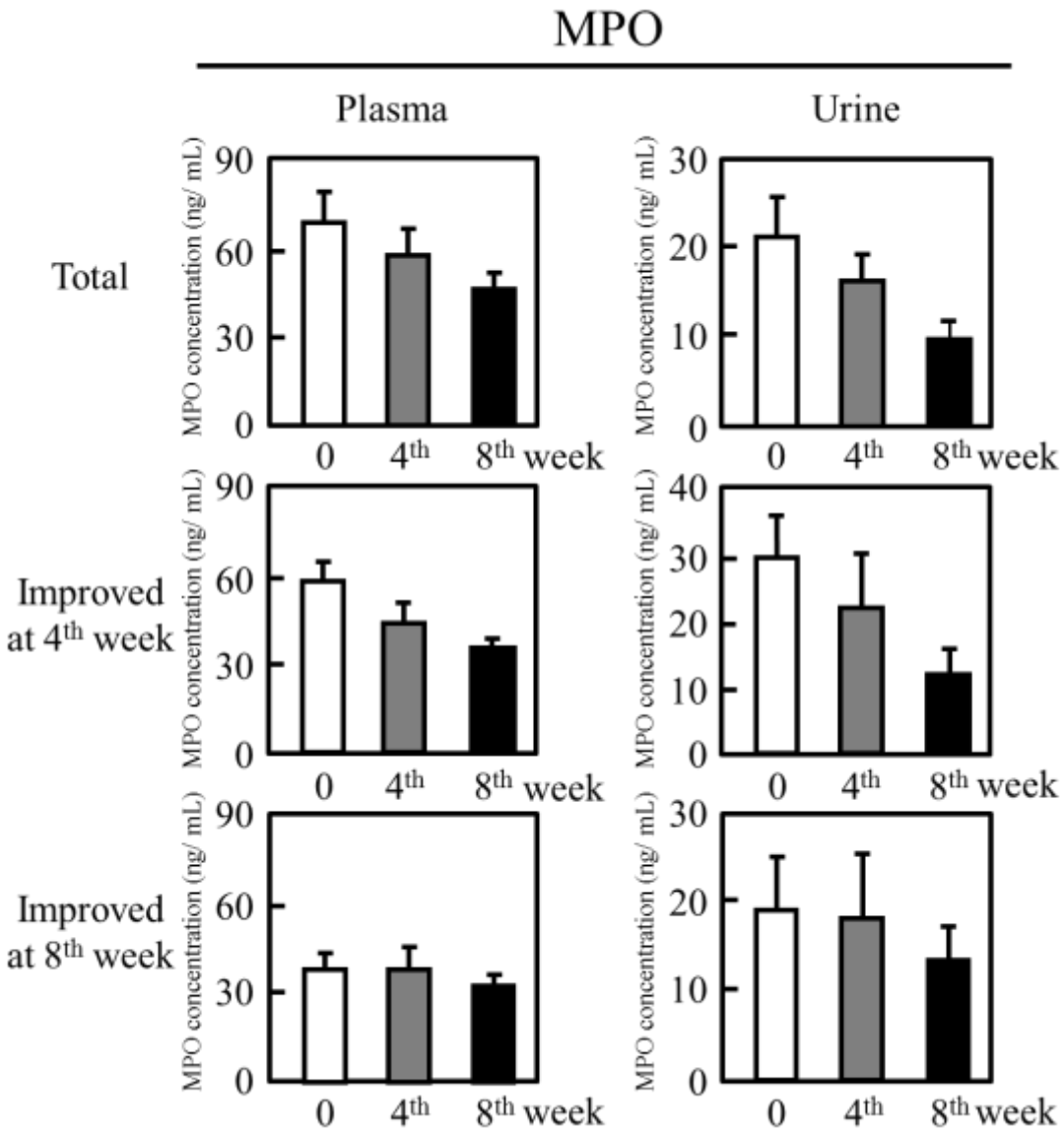


Figure 4.2C. The effect of black soybean consumption on biomarkers related to MPO concentrations in the plasma and urine. MPO was measured using corresponding kit and represented as means ± standard deviation. *$p < 0.05$ *vs.* 0 week, *Dunnett`s* Test. Results of 44 volunteers were denoted as 'Total', and results of volunteers who showed reversed vascular age at the 4th or 8th week were denoted as 'Improved at the 4th week' and 'Improved at the 8th week', respectively.

4.3.5. Increased polyphenol concentration in the plasma and urine

Using the analytical method developed in Chapter 2 with some modifications, 12 phenolic compounds in human plasma and urine were investigated (Figure 4.3, Table 4.4).

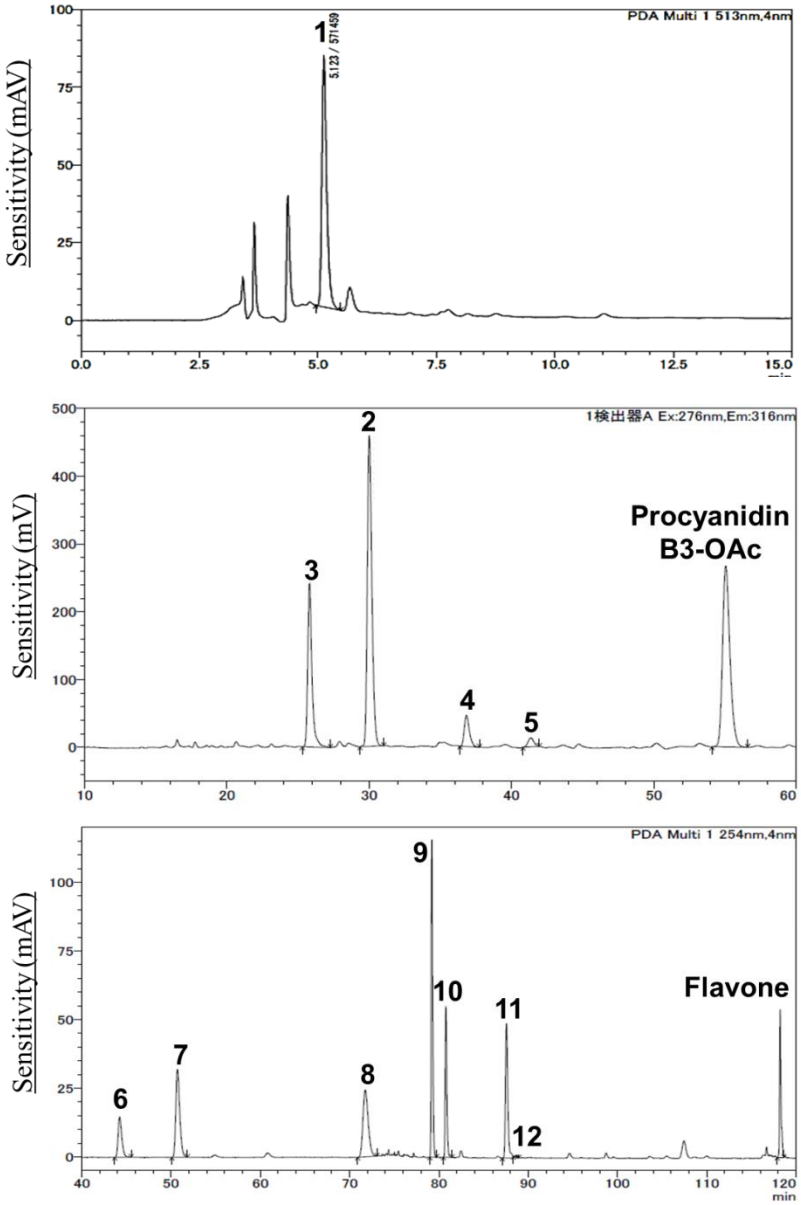


Figure 4.3.
Chromatograph of the authentic compound of
(1)cyanidin-3-O-glucoside,
(2)(−)-epicatechin,
(3)procyanidin B2,
(4)procyanidin C1,
(5)cinnamtannin A2,
(6)daidzin,
(7)glycitin,
(8)genistin,
(9)daizein,
(10)glycitein,
(11)genistein,
(12)equol.
Procyanidin B3-OAc and flavone were used as the internal standard for flavan-3-ols and isoflavones, respectively.

Table 4.4. Calibration curves of black soybean polyphenols in urine and plasma

Polyphenols	Plasma		Urine	
	Equation	R^2	Equation	R^2
Cyanidin-3-*O*-glucoside	y=1E+06x	0.9948	y=635850x	0.9997
(−)-Epicatechin	y=0.0681x	0.9942	y=0.0058x	0.9891
Procyanidin B2	y=0.0463x	0.9723	y=0.0015x	0.9875
Procyanidin C1	y=0.0634x	0.9926	y=0.0046x	0.9875
Cinnamtannin A2	y=0.0406x	0.9468	y=0.0044x	0.9665
Daidzin	y=17.847x	0.9971	y=8.7231x	0.9977
Glycitin	y=39.697x	0.9981	y=24.305x	0.9922
Genistin	y=43.811x	0.9982	y=28.339x	0.9875
Daidzein	y=53.831x	0.9804	y=43.437x	0.9951
Glycitein	y=26.593x	0.9988	y=25.811x	0.9699
Genistein	y=36.432x	0.9987	y=24.797x	0.9859
Equol	y=7.8599x	0.9986	y=5.6583x	0.9782

y is represented as the peak area ratio of flavan-3-ol and internal standard, and x is represented as concentration, μM.

Increased polyphenol concentrations in the plasma (Table 4.5A) and urine (Table 4.5B) of 'Total' group were observed. Polyphenols detected without enzymatic hydrolysis were denoted as 'Free', and polyphenols detected after the enzymatic hydrolysis were denoted as 'Total'. Increases without significance were observed in most of the polyphenols except for cyanidin-3-*O*-glucoside in the urine, which was not detected in the plasma and significantly increased at the 4th week. Significant increases were observed in the sum amount of 12 polyphenols at the 4th and 8th week in both plasma and urine, and it was 2- to 3-fold higher than that at the 0 week.

In 'Improved at the 4th week' group and 'Improved at the 8th week' group, significant increases in certain polyphenols were also observed (Table 4.6, Table 4.7). In 'Improved at the 4th week' group, 10 out of 12 polyphenols significantly increased in the plasma (Table 4.6A) and/or the urine (Table 4.6B) at the 4th week of the trial, including cyanidin-3-*O*-glucoside, (−)-epicatechin, procyanidin B2, procyanidin C1, cinnamtannin A2, daidzin, daidzein, glycitin, glycitein and genistein; and it decreased to 5 polyphenols (procyanidin C1, cinnamtannin A2, daidzein, genistin and genistein) at

the 8th week. As to ‘Improved at the 8th week’ group, 8 polyphenols (cyanidin-3-*O*-glucoside, procyanidin B2, procyanidin C1, daidzin, daidzein, glycitin, glycitein and genistein significantly increased in the plasma (Table 4.7A) and/or the urine (Table 4.7B) at the 4th week of the trial; and it decreased to 6 polyphenols (procyanidin B2, procyanidin C1, cinnamtannin A2, daidzein, genistin and genistein) at the 8th week. Procyanidin B2, procyanidin C1, cinnamtannin A2, daidzein and genistein were most increased polyphenols (2- to3-fold to that at 0 week) observed during the trial ($p<0.01$).

Table 4.5A. 12 polyphenols measured in plasma of 44 volunteers during 8-week consumption of roasted black soybean

Polyphenols	Plasma					
	0 week		4th week		8th week	
	Free	Total	Free	Total	Free	Total
Cyanidin-3-*O*-glucoside	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
(−)-Epicatechin	0.048±0.033	0.066±0.059	0.049±0.033	0.072±0.060	0.061±0.053	0.085±0.087
Procyanidin B2	0.013±0.009	0.015±0.009	0.023±0.015	0.022±0.016	0.027±0.016	0.028±0.017
Procyanidin C1	0.001±0.001	0.001±0.001	0.001±0.002	0.002±0.002	0.002±0.002	0.003±0.003
Cinnamtannin A2	0.002±0.002	0.002±0.002	0.003±0.003	0.004±0.003	0.004±0.004	0.004±0.003
Daidzin	0.012±0.006	0.013±0.011	0.018±0.010	0.020±0.009	0.018±0.012	0.018±0.007
Daidzein	0.010±0.005	0.011±0.007	0.013±0.007	0.016±0.010	0.012±0.008	0.013±0.010
Glycitin	0.014±0.007	0.016±0.010	0.021±0.014	0.029±0.022	0.020±0.015	0.021±0.017
Glycitein	0.024±0.007	0.027±0.008	0.026±0.009	0.029±0.009	0.026±0.009	0.028±0.009
Genistin	0.007±0.010	0.008±0.011	0.008±0.010	0.010±0.010	0.008±0.010	0.012±0.013
Genistein	0.034±0.021	0.038±0.025	0.041±0.019	0.047±0.025	0.039±0.017	0.040±0.026
Equol	0.008±0.006	0.011±0.012	0.010±0.009	0.016±0.012	0.008±0.008	0.013±0.014
Sum	0.164±0.059	0.195±0.064	0.209±0.066**	0.261±0.092##	0.210±0.073**	0.268±0.104##

Means ± SE are shown, μmol/L. *, #$p < 0.05$, **, ##$p < 0.01$. * and ** represent for significant differences from free compound of 0 week. # and ## represent for that from total compound of 0 week. *Dunnett`s* Test.

Table 4.5B. 12 polyphenols measured in urine of 44 volunteers during 8-week consumption of roasted black soybean

Polyphenols	Urine					
	0 week		**4th week**		**8th week**	
	Free	Total	Free	Total	Free	Total
Cyanidin-3-*O*-glucoside	0.009±0.009	0.011±0.010	0.016±0.012*	0.020±0.014$^{\#}$	0.013±0.010	0.015±0.011
(−)-Epicatechin	0.038±0.059	0.062±0.049	0.088±0.084	0.165±0.144	0.067±0.063	0.127±0.120
Procyanidin B2	0.118±0.137	0.144±0.124	0.234±0.166	0.262±0.194	0.219±0.154	0.264±0.231
Procyanidin C1	0.008±0.009	0.008±0.010	0.014±0.012	0.019±0.016	0.012±0.011	0.016±0.015
Cinnamtannin A2	0.002±0.003	0.004±0.004	0.009±0.009	0.010±0.008	0.008±0.008	0.008±0.009
Daidzin	0.055±0.062	0.071±0.125	0.107±0.122	0.147±0.163	0.122±0.323	0.175±0.243
Daidzein	0.596±0.770	4.741±3.595	1.718±1.994	8.387±4.574	1.992±2.180	9.961±6.744
Glycitin	0.041±0.041	0.057±0.123	0.048±0.041	0.083±0.086	0.061±0.055	0.093±0.125
Glycitein	0.263±0.391	1.034±1.380	0.394±0.507	2.152±1.703	0.487±0.463	2.468±2.624
Genistin	0.078±0.112	0.172±0.231	0.124±0.169	0.327±0.396	0.180±0.252	0.450±0.460
Genistein	0.397±0.451	5.319±6.086	1.100±1.172	11.891±9.831	1.443±2.066	14.949±11.34
Equol	0.146±0.116	0.443±0.905	0.374±1.433	3.439±6.942	1.439±7.390	5.418±14.54
Sum	2.597±2.579	10.978±9.954	4.470±3.897	30.770±24.57$^{\#\#}$	5.685±5.618**	29.237±26.73$^{\#\#}$

Means ± SE are shown, μmol/L. *, $^{\#}p < 0.05$, **, $^{\#\#}p < 0.01$. * and ** represent for significant differences from free compound of 0 week. $^{\#}$ and $^{\#\#}$ represent for that from total compound of 0 week. *Dunnett`s* Test

Table 4.6A. 12 polyphenols measured in plasma of 25 volunteers whose improved vascular function was observed at the 4th week during 8-week consumption of roasted black soybean

Polyphenols	Plasma					
	0 week		4th week		8th week	
	Free	Total	Free	Total	Free	Total
Cyanidin-3-*O*-glucoside	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
(−)-Epicatechin	0.048±0.032	0.077±0.070	0.052±0.037	0.082±0.062	0.062±0.057	0.096±0.078
Procyanidin B2	0.013±0.010	0.014±0.008	0.021±0.016	0.023±0.019##	0.024±0.015	0.028±0.014
Procyanidin C1	0.001±0.001	0.001±0.001	0.001±0.001	0.002±0.002	0.001±0.001*	0.002±0.001
Cinnamtannin A2	0.001±0.002	0.002±0.002	0.001±0.002	0.003±0.003	0.003±0.004*	0.004±0.003
Daidzin	0.012±0.006	0.013±0.011	0.017±0.009	0.021±0.009#	0.017±0.011	0.018±0.007
Daidzein	0.009±0.005	0.010±0.007	0.014±0.007*	0.017±0.011##	0.011±0.007	0.012±0.007
Glycitin	0.014±0.006	0.014±0.009	0.021±0.014*	0.026±0.021#	0.017±0.007	0.015±0.009
Glycitein	0.024±0.007	0.024±0.009	0.025±0.009	0.030±0.009#	0.026±0.008	0.028±0.010
Genistin	0.004±0.006	0.008±0.012	0.006±0.006	0.011±0.012	0.006±0.006	0.011±0.015
Genistein	0.037±0.023	0.044±0.031	0.046±0.016	0.054±0.027	0.040±0.017	0.044±0.028
Equol	0.007±0.006	0.011±0.011	0.010±0.009	0.016±0.013	0.012±0.006	0.014±0.014
Sum	0.168±0.060	0.203±0.070	0.211±0.064	0.279±0.102#	0.211±0.073	0.268±0.101#

Means ± SE are shown, μmol/L. *, #$p < 0.05$, **, ##$p < 0.01$. * and ** represent for significant differences from free compound of 0 week. # and ## represent for that from total compound of 0 week. *Dunnett`s* Test.

Table 4.6B. 12 polyphenols measured in urine of 25 volunteers whose improved vascular function was observed at the 4th week during 8-week consumption of roasted black soybean

Polyphenols	Urine					
	0 week		4th week		8th week	
	Free	Total	Free	Total	Free	Total
Cyanidin-3-*O*-glucoside	0.009±0.007	0.012±0.009	0.015±0.010*	0.019±0.013$^{\#}$	0.011±0.005	0.014±0.006
(−)-Epicatechin	0.024±0.042	0.136±0.242	0.175±0.253*	0.460±0.472$^{\#}$	0.123±0.173	0.221±0.423
Procyanidin B2	0.217±0.294	0.191±0.229	0.505±0.576	0.587±1.120	0.406±0.342	0.368±0.315
Procyanidin C1	0.008±0.009	0.010±0.010	0.019±0.017*	0.029±0.027$^{\#\#}$	0.014±0.012	0.019±0.015
Cinnamtannin A2	0.005±0.009	0.005±0.009	0.012±0.013*	0.018±0.017$^{\#\#}$	0.008±0.008	0.009±0.009
Daidzin	0.043±0.030	0.052±0.065	0.105±0.120*	0.142±0.190	0.047±0.041	0.098±0.106
Daidzein	1.396±2.373	3.741±3.360	3.033±6.957	11.375±8.408$^{\#\#}$	3.137±3.693	8.364±7.237$^{\#}$
Glycitin	0.041±0.035	0.070±0.058	0.045±0.030	0.083±0.092	0.057±0.092	0.077±0.077
Glycitein	0.329±0.505	1.124±1.479	0.446±0.612	3.006±3.226	0.532±0.543	3.430±6.131
Genistin	0.047±0.053	0.127±0.153	0.119±0.176	0.267±0.186	0.185±0.315	0.330±0.307$^{\#}$
Genistein	0.522±0.516	5.202±4.765	1.104±1.109	16.973±15.99$^{\#\#}$	1.705±2.524*	13.153±12.31
Equol	0.163±0.120	0.590±1.128	0.160±0.217	2.386±4.763	0.453±1.341	5.613±15.05
Sum	2.482±2.225	10.926±8.558	4.080±3.400	31.761±20.98$^{\#\#}$	6.232±6.092**	32.295±32.34$^{\#\#}$

Means ± SE are shown, μmol/L. *, $^{\#}p < 0.05$, **, $^{\#\#}p < 0.01$. * and ** represent for significant differences from free compound of 0 week. $^{\#}$ and $^{\#\#}$ represent for that from total compound of 0 week. *Dunnett`s* Test.

Table 4.7A. 12 polyphenols measured in plasma of 26 volunteers whose improved vascular function was observed at the 8th week during 8-week consumption of roasted black soybean

Polyphenols	Plasma					
	0 week		4th week		8th week	
	Free	Total	Free	Total	Free	Total
Cyanidin-3-*O*-glucoside	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
(−)-Epicatechin	0.042±0.022	0.063±0.053	0.050±0.033	0.084±0.069	0.058±0.048	0.104±0.097
Procyanidin B2	0.014±0.008	0.013±0.008	0.025±0.013*	0.025±0.015##	0.028±0.013**	0.029±0.015##
Procyanidin C1	0.001±0.001	0.001±0.001	0.001±0.001	0.002±0.002	0.002±0.002*	0.003±0.003#
Cinnamtannin A2	0.002±0.002	0.002±0.002	0.003±0.003	0.003±0.004	0.004±0.005	0.005±0.003##
Daidzin	0.012±0.006	0.015±0.013	0.019±0.010*	0.021±0.009#	0.020±0.014	0.020±0.007
Daidzein	0.010±0.005	0.011±0.007	0.014±0.006*	0.016±0.009#	0.012±0.006	0.013±0.008
Glycitin	0.016±0.006	0.018±0.010	0.023±0.014##	0.026±0.015	0.018±0.007	0.019±0.017
Glycitein	0.026±0.006	0.026±0.007	0.028±0.009	0.031±0.008	0.027±0.009	0.030±0.008
Genistin	0.006±0.007	0.006±0.007	0.006±0.008	0.013±0.013	0.005±0.006	0.010±0.014
Genistein	0.037±0.023	0.039±0.030	0.041±0.018	0.048±0.026	0.038±0.018	0.039±0.025
Equol	0.009±0.006	0.013±0.012	0.013±0.011	0.015±0.013	0.010±0.004	0.014±0.013
Sum	0.170±0.050	0.207±0.068	0.222±0.059*	0.282±0.088##	0.225±0.070**	0.293±0.103##

Means ± SE are shown, μmol/L. *, #$p < 0.05$, **, ##$p < 0.01$. * and ** represent for significant differences from free compound of 0 week. # and ## represent for that from total compound of 0 week. *Dunnett`s* Test.

Table 4.7B. 12 polyphenols measured in urine of 26 volunteers whose improved vascular function was observed at the 8th week during 8-week consumption of roasted black soybean

Polyphenols	Urine					
	0 week		4th week		8th week	
	Free	Total	Free	Total	Free	Total
Cyanidin-3-*O*-glucoside	0.010±0.008	0.012±0.009	0.015±0.010	0.020±0.012#	0.012±0.008	0.015±0.009
(−)-Epicatechin	0.076±0.151	0.110±0.158	0.125±0.180	0.240±0.282	0.096±0.129	0.125±0.120
Procyanidin B2	0.197±0.269	0.179±0.194	0.545±0.582*	0.687±1.158#	0.473±0.408	0.494±0.466
Procyanidin C1	0.010±0.015	0.012±0.016	0.017±0.015	0.028±0.028#	0.014±0.010	0.016±0.014
Cinnamtannin A2	0.002±0.003	0.003±0.004	0.010±0.010**	0.013±0.010##	0.008±0.007*	0.008±0.008#
Daidzin	0.044±0.023	0.046±0.055	0.106±0.134*	0.139±0.130##	0.057±0.056	0.107±0.122
Daidzein	1.410±2.396	3.344±3.499	3.429±7.049	10.805±8.983##	3.627±4.832	8.360±7.339#
Glycitin	0.035±0.034	0.065±0.059	0.040±0.026	0.065±0.064	0.058±0.064	0.092±0.144
Glycitein	0.371±0.517	1.034±1.486	0.494±0.628	2.913±3.338#	0.787±1.261	2.573±3.003
Genistin	0.057±0.083	0.103±0.150	0.094±0.129	0.268±0.201	0.252±0.359*	0.419±0.427##
Genistein	0.419±0.444	5.268±6.528	1.355±1.380	15.248±16.25##	1.600±2.564*	11.414±10.37
Equol	0.157±0.116	0.481±1.113	0.161±0.216	2.442±6.531	2.411±9.691	6.597±17.07
Sum	1.852±1.510	9.210±9.467	4.610±4.194	27.912±22.10#	5.552±5.804**	28.273±29.46##

Means ± SE are shown, μmol/L. *, #$p < 0.05$, **, ##$p < 0.01$. * and ** represent for significant differences from free compound of 0 week. # and ## represent for that from total compound of 0 week. *Dunnett`s* Test.

4.3.6. Correlation between physiological concentration of polyphenols and biomarkers

The correlation between polyphenol concentration and measured biomarkers was analyzed using Pearson correlation coefficient. Eleven out of 12 polyphenols was found significantly correlated to at least one biomarker that related to the vascular function (Figure 4.4A). Among these polyphenols, total procyanidin B2 in the plasma and total cinnamtannin A2 in the urine of 'Improved at the 4th week' group were found most correlated to these biomarkers. Negative correlation was observed between total procyanidin B2 and vascular age (r=−0.323, p=0.032), *b* wave (r=−0.448, p=0.002), height ratio of *b/a* (r=−0.424, p=0.004), systolic blood pressure (r=−0.418, p=0.005) and diastolic blood pressure (r=−0.379, p=0.011). Positive correlation was observed between total cinnamtannin A2 and biological age (r=0.570, p<0.001), vascular age (r=0.348, p=0.021), *b* wave (r=0.311, p=0.040), height ratio of *b/a* (r=0.292, p=0.040) and systolic blood pressure (r=0.317, p=0.036).

Negative correlation between polyphenols and oxidative stress was observed in Figure 4.4B. 8-OHdG was most negatively correlated to free daidzin (r=−0.310, p=0.041), free glycitin (r=−0.329, p=0.029), total glycitein (r=−0.302, p=0.046), free equol (r=−0.325, p=0.031) and total procyanidin C1(r=−0.333, p=0.027). Significant negative correlation was also found between HEL, MPO, NO and other polyphenols, but strong positive correlation was observed between HEL and free (−)-epicatechin (r=0.596, p<0.001) as well as total (−)-epicatechin (r=0.697, p<0.001).

In addition to the biomarkers of vascular function and oxidative stress, negative correlation was observed between most polyphenols and the biomarkers that related to the risk of obese, including significant correlation between free glycitin and body fat (r=−0.345, p=0.022), total glycitin and body weight (r=−0.301, p=0.047), as well as free genistein and body weight (r=−0.346, p=0.021) (Figure 4.4C). Positive correlation was also observed in certain polyphenols, including total cinnamtannin A2 in the urine

of ‘Improved at the 4th week’ group which was found significantly correlated to body weight (r=0.311, p=0.003), BMI (r=0.548, p<0.001), body fat (r=0.435, p=0.003) and visceral fat (r=0.311, p<0.001).

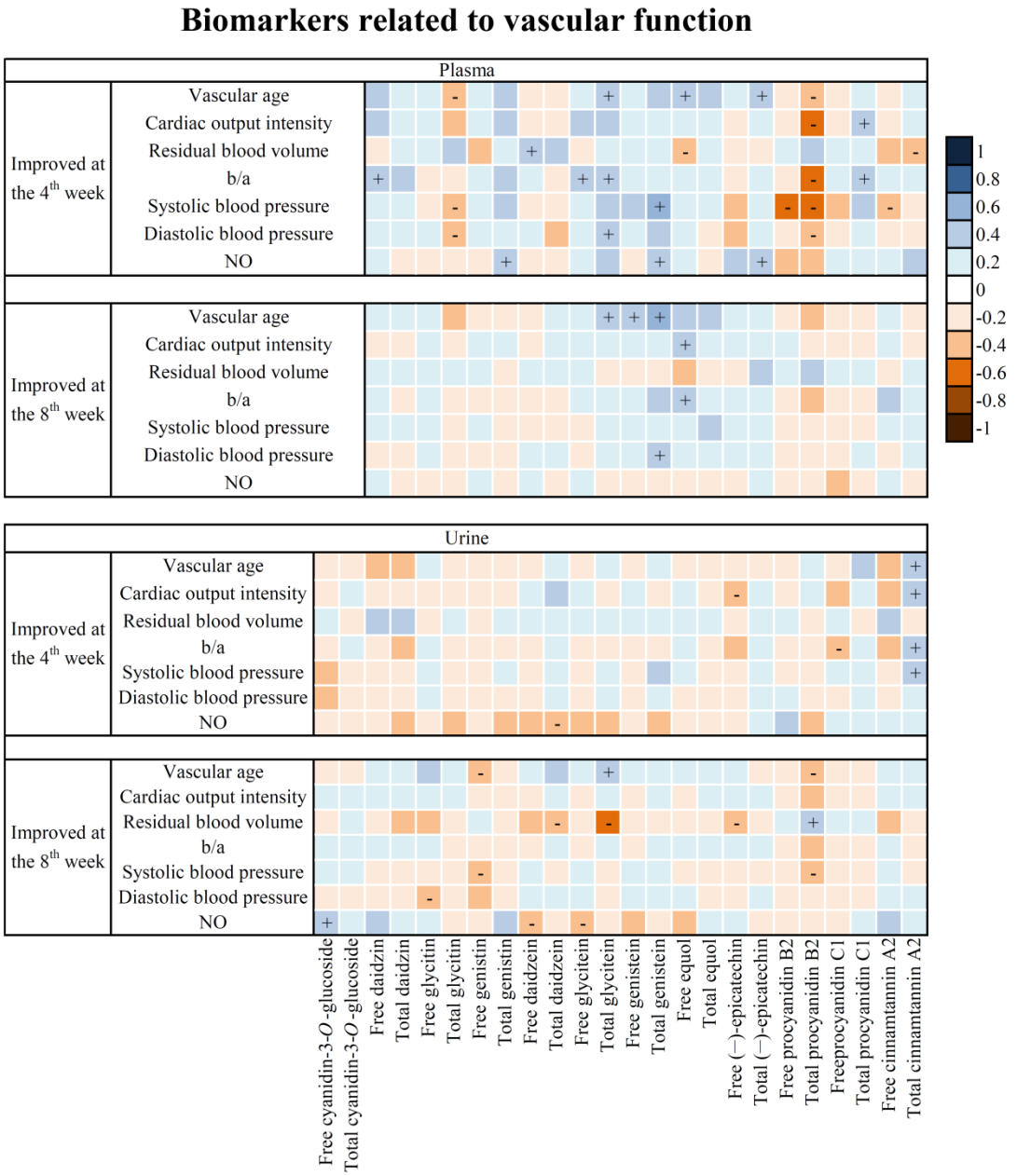


Figure 4.4A. An association heat map between physiological concentrations of polyphenols and biomarkers of vascular function. Each square indicates the Pearson correlation coefficient vales of corresponding factors. Positive correlations were shown in blue, negative ones were shown in red and significant results were marked with ‘+’ and ‘−’, respectively, p<0.05, t-test.

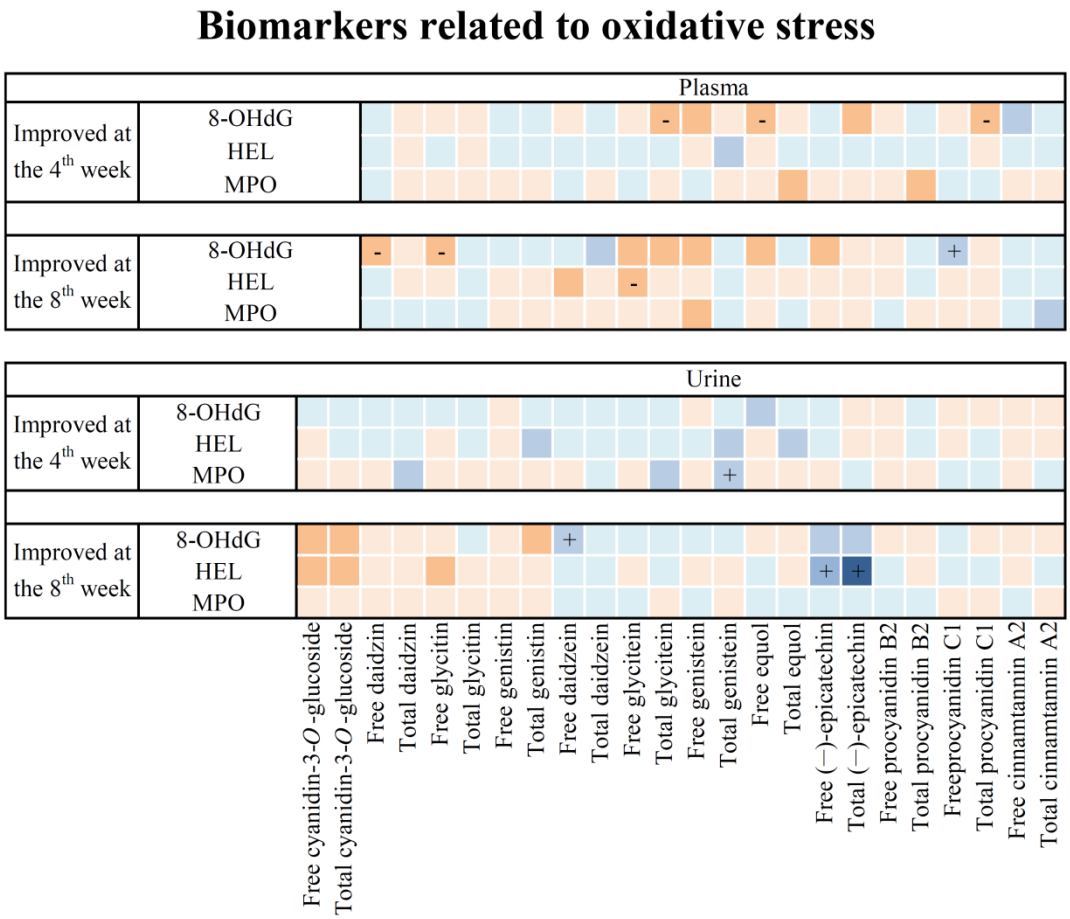


Figure 4.4B. An association heat map between physiological concentrations of polyphenols and biomarkers of oxidative stress. Each square indicates the Pearson correlation coefficient vales of corresponding factors. Positive correlations were shown in blue, negative ones were shown in red and significant results were marked with '+' and '−', respectively, $p<0.05$, *t*-test.

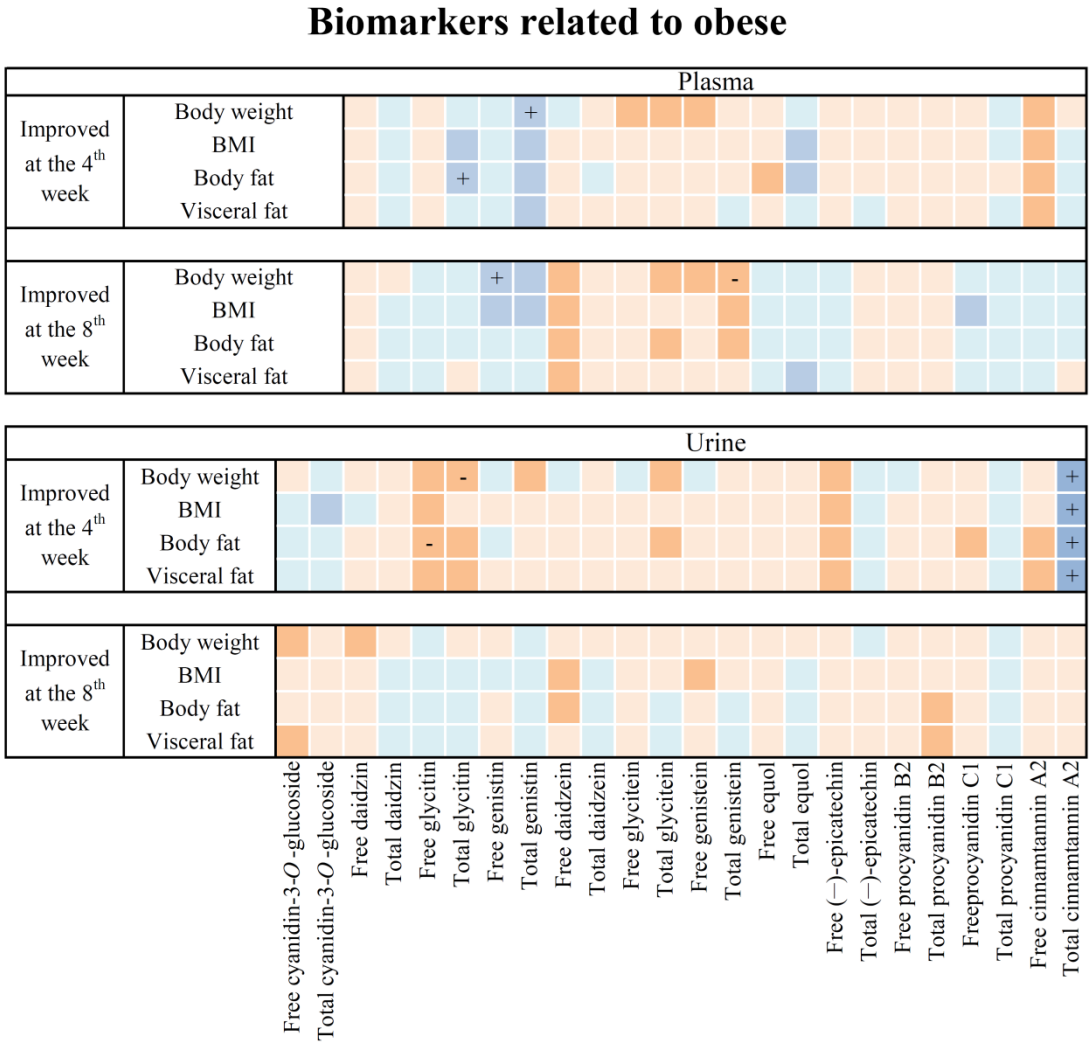


Figure 4.4C. An association heat map between physiological concentrations of polyphenols and biomarkers of obese. Each square indicates the Pearson correlation coefficient vales of corresponding factors. Positive correlations were shown in blue, negative ones were shown in red and significant results were marked with '+' and '−', respectively, $p<0.05$, *t*-test.

4.3.7. Discussion

In this chapter, significantly improved vascular function, including lowered vascular age and improved APG during 8-week consumption of roasted black soybeans (Table 4.3), was observed in human during an 8-week consumption of roasted black soybeans. Vascular age is one of the common indicators that predict the risk of cardiovascular diseases by age, and it performs better than the chronological age of volunteers [102]. Lowered vascular age was observed in 33 out 44 volunteers who had higher vascular age than their chronological age before the trial began. Although the vascular age of volunteers was still higher than their chronological age at the end of the trial, continuous effect was expected with a sustained consumption of roasted black soybeans. Compared to the vascular age, APG provides more detailed information about the health condition of blood vessels, including cardiac output intensity (*b* wave), residual blood volume (*c* wave) and vascular compliance (*d* wave). In addition, the height ratios of each wave to *a* wave (*b/a*, *c/a* and *d/a*) are typical indicators used in APG for assessing the arterial stiffness [103, 104]. In this study, significantly increased cardiac output intensity, reduced residual blood volume and vascular compliance were observed during the trial, indicating black soybean consumption improved the arterial stiffness. Improvement in the arterial stiffness was also reported in healthy adults after 6-week supplementation of isoflavones (genistein and daidzein) [105], in patients with coronary artery disease after 4-week consumption of cranberry juice [106], and in healthy males after 1-week consumption of black tea [107]. Although these improvements in arterial stiffness were measured by pulse wave velocity (PWV) or flow-mediated dilation (FMD) which was different from the relatively new method used in this study, coincide results were observed. NO is another indicator to evaluate the vascular function. It contributes to the relaxation of blood vessels and eventually leads to the improvement in the arterial stiffness [108]. Significantly increased NO (Figure 4.4A) suggested that black soybean consumption improved arterial stiffness by

increasing NO in human. Coincide results were reported that NO was increased in the plasma and urine of healthy human with improved arterial stiffness after the ingestion of a cocoa drink [109]. In addition, more clearly increased NO was observed in 'Improved at the 4th week' and 'Improved at the 8th week' group compared to 'Total' group (Fig. 2), furtherly suggesting that black soybean consumption improved vascular function by increasing NO in human.

Underlying mechanism of vascular dysfunction is closely related to oxidative stress which leads to the onset of cardiovascular diseases [110]. Representative biomarkers of oxidative stress including 8-OHdG, HEL and MPO changed significantly in the plasma and/or urine after black soybean consumption (Figure 4.2), resulting in the ameliorated oxidative stress. These indicators contribute to different aspects of the oxidative stress. Decrease in 8-OHdG (Figure 4.2A) contributed to the ameliorated DNA damage which was reported triggering the development of cardiovascular diseases and type II diabetes [111]. Strongly decreased 8-OHdG was also reported in human after a supplementation of red wine during a 3-month high fat diet and Mediterranean diet [112]. Decrease in HEL (Figure 4.2B) and MPO (Figure 4.2C) contributed to improved lipid peroxidation [113, 114], and similar results were reported in human ingested cocoa powder for 12 weeks [115]. Similar as the results of NO (Figure 4.1), more clearly decreased 8-OHdG (Figure 4.2A) was observed in 'Improved at the 4th week' and 'Improved at the 8th week' group. This result suggested that black soybean consumption improved vascular function by regulating oxidative stress in human.

Polyphenols are usually considered responsible for the beneficial effects exerted by food materials [105-107, 109, 112, 115], but only few human studies have reported the physiological concentrations of corresponding polyphenols after the consumption of test materials. In this study, increased physiological concentrations of 11 black soybean polyphenols, and 1 intestinal bacterial metabolite of daizdzein, namely equol, were observed after the consumption of roasted black soybeans, but significant increase was

only observed in their sum amount (Table 4.5). Lack of restrictions on the diet throughout the trial, and individual differences in the polyphenol metabolism were considered as the possible reasons. Surprisingly, after the results were stratified into 'Improved at the 4th week' group and 'Improved at the 8th week' group, significant increases in the corresponding polyphenols were observed (Table 4.6, Table 4.7). These results suggested the direct effect of black soybean consumption on increased physiological concentrations of related polyphenols. Similar results were also reported by previous studies: Schroeter *et al.* reported that (−)-epicatechin, (+)-catechin, their related metabolites and the sum flavanols in the plasma increased greatly within 6 h after ingestion of flavanol-rich cocoa [109]; Urquiaga *et al.* reported that total plasma and urine polyphenols increased during the supplementation with red wine [112]. These results supported that dietary intake of black soybean was an effective way to increase polyphenol concentrations in the body. Polyphenol concentration in the plasma and urine of volunteers whose vascular age did not change throughout the trial or became higher at the end of the trial were also measured, but significant increase was not observed (Figure 4.S1), indicating the direct relation between the increased polyphenol concentration and the lowered vascular age. Further analysis using Pearson correlation coefficient showed that there was a significant correlation between the increased polyphenol concentration and improved vascular function, oxidative stress and even risk of obese (Figure 4.4). In general, these results suggested that volunteers with higher physiological concentrations of polyphenols had more improved vascular function, oxidative stress and lower risk of obese after black soybean consumption. Body weight, body fat mass (Table 4.1) and blood pressure (Table 4.3) were not significantly changed as absolute amount, but they were found negatively correlated to polyphenol concentrations (Figure 4.4B-C), indicating the potential benefits of black soybean consumption on these indicators. Certain polyphenols seemed to increase these risks, such as total cinnamtannin A2 positively correlating to the vascular function (Figure

4.4A) and the risk of obese (Figure 4.4C), but when the correlation was analyzed using $\Delta_{\text{polyphenol concentration}}$ (the increase compared to 0 week) and $\Delta_{\text{biomarker}}$, significant correlation was no longer observed between total cinnamtannin A2 and the body weight (r=−0.090, p=0.561), BMI (r=−0.107, p=0.489), body fat (r=0.037, p=0.812) and visceral fat (r=0.011, p=0.944). Same calculation was performed to other results suggesting increased polyphenol concentrations did not increased these risks, except that the correlation between HEL and free (−)-epicatechin (r=0.390, p=0.009), and total (−)-epicatechin (r=0.515, p<0.001) in the urine of 'Improved at 8th week' group was still significantly positive. However, the HEL in the urine was not significantly increased in the corresponding group, suggesting that increased (−)-epicatechin by black soybean consumption in this study was unlikely to affect the HEL level greatly enough to increase the risk of oxidative stress. Other correlations between the polyphenols and biomarkers were also calculated and shown in Figure 4.S2.

Besides not using a double-blind cross-over trial, restrictions on the diet were not conducted in this study. It is usually considered more accurate to assess the beneficial effects of food consumption with restrictions on the diet, but some of the restrictions in the previous studies were hardly achieved in daily life, such as abstaining from coffee and green tea before and during the trial which lasted for 7 weeks [116], or forbidden consumption of berries, wine and all related products in the study of strawberry and cranberry polyphenols [117]. Volunteers in this study were asked to keep their usual diet before and during the experiment period, and take the black soybeans consumption at any time with any amount as long as 30 g/day of the test material was consumed, so that the possible consumption and its related beneficial effects with an ordinary diet was investigated. Even with the possible interference from the daily diet, beneficial effects of 8-week black soybean consumption on vascular function and oxidative stress correlated with increased physiological concentrations of polyphenols with minor improvement in the HRQOL (Figure 4.S3) were observed.

4.3.8. Conclusions

Consumption of roasted black soybeans with an intake of 30 g/day for 8 weeks significantly improved the vascular function and oxidative stress. The black soybean consumption also increased the physiological concentrations of polyphenols in the plasma and urine, which contributed to the improved vascular function and oxidative stress leading to the lowered risk of CVD in human.

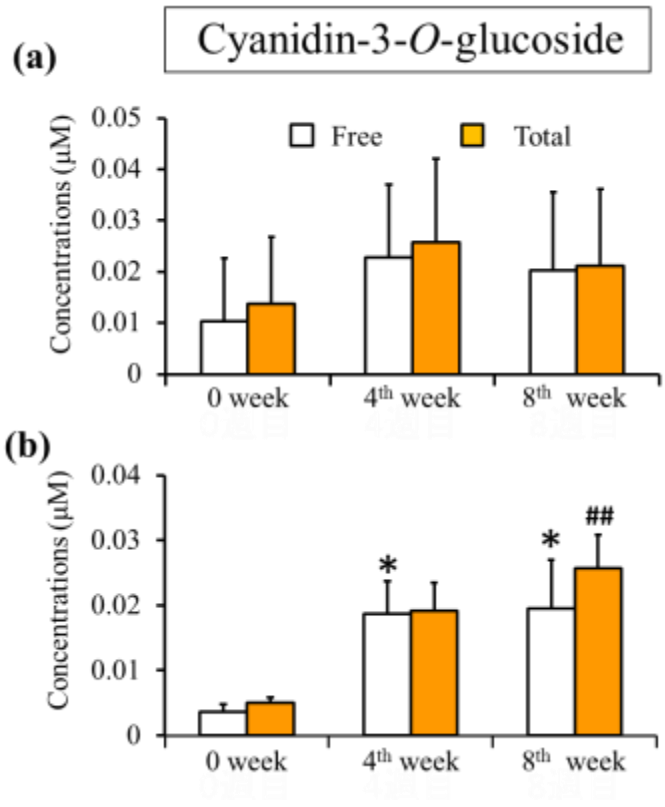


Figure 4. S1A. Concentrations of cyanidin-3-*O*-glucoside in the urine of volunteers whose vascular age did not change throughout the trial (a) and volunteers whose vascular age slightly increased during the trial (b). Results are represented as means±SE. *, #$p < 0.05$, **, ##$p < 0.01$. * and ** represent for significant differences from values of free compounds of the 0 week. # and ## represent for significant differences from values of total compounds of the 0 week group, determined by Dunnett`s Test.

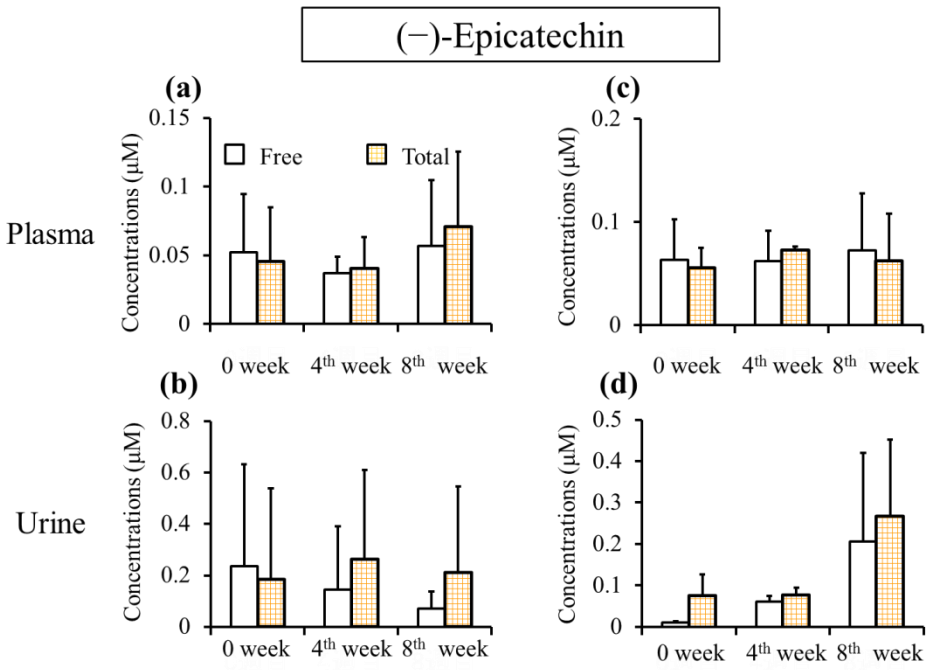


Figure 4. S1B. Concentrations of (−)-epicatechin in the plasma and urine of volunteers whose vascular age did not change throughout the trial (a, b) and volunteers whose vascular age slightly increased during the trial (c, d). Results are represented as means±SE.

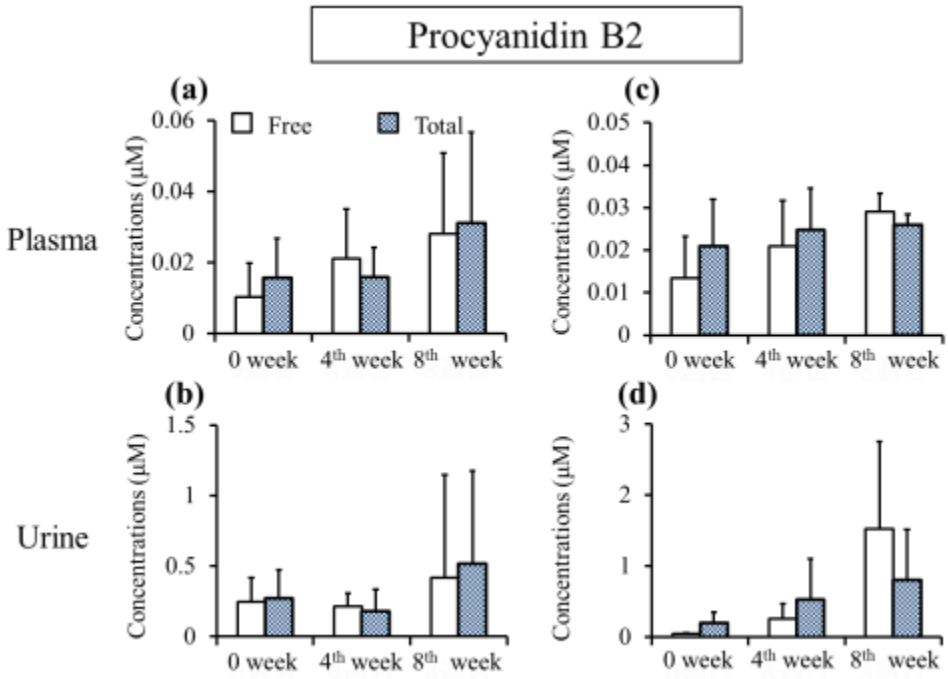


Figure 4. S1C. Concentrations of procyanidin B2 in the plasma and urine of volunteers whose vascular age did not change throughout the trial (a, b) and volunteers whose vascular age slightly increased during the trial (c, d). Results are represented as means±SE.

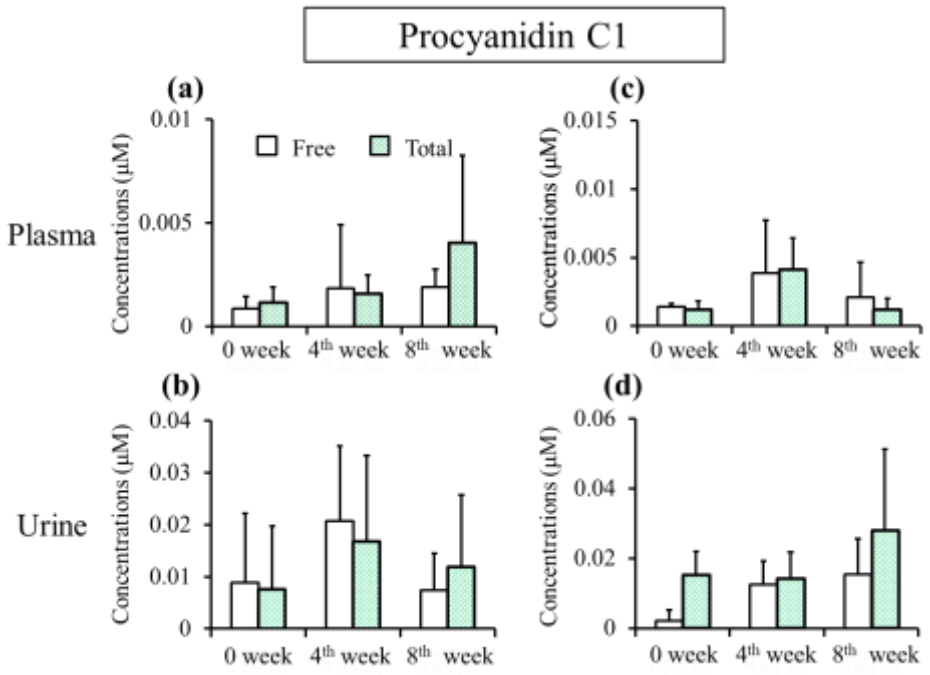


Figure 4. S1D. Concentrations of procyanidin C1 in the plasma and urine of volunteers whose vascular age did not change throughout the trial (a, b) and volunteers whose vascular age slightly increased during the trial (c, d). Results are represented as means±SE.

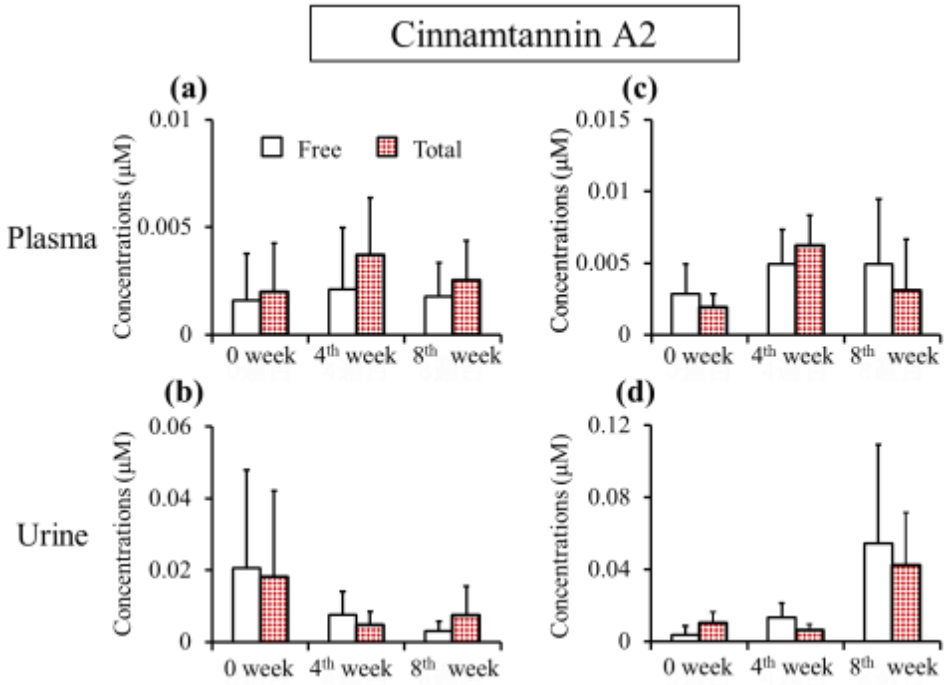


Figure 4. S1D. Concentrations of cinnamtannin A2 in the plasma and urine of volunteers whose vascular age did not change throughout the trial (a, b) and volunteers whose vascular age slightly increased during the trial (c, d). Results are represented as means±SE.

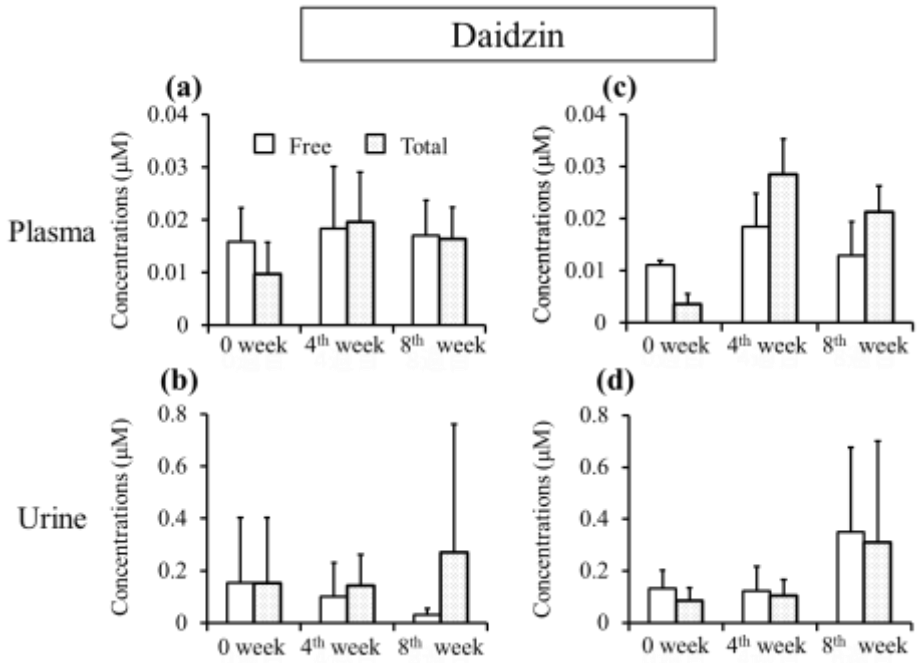


Figure 4. S1E. Concentrations of daidzin in the plasma and urine of volunteers whose vascular age did not change throughout the trial (a, b) and volunteers whose vascular age slightly increased during the trial (c, d). Results are represented as means±SE.

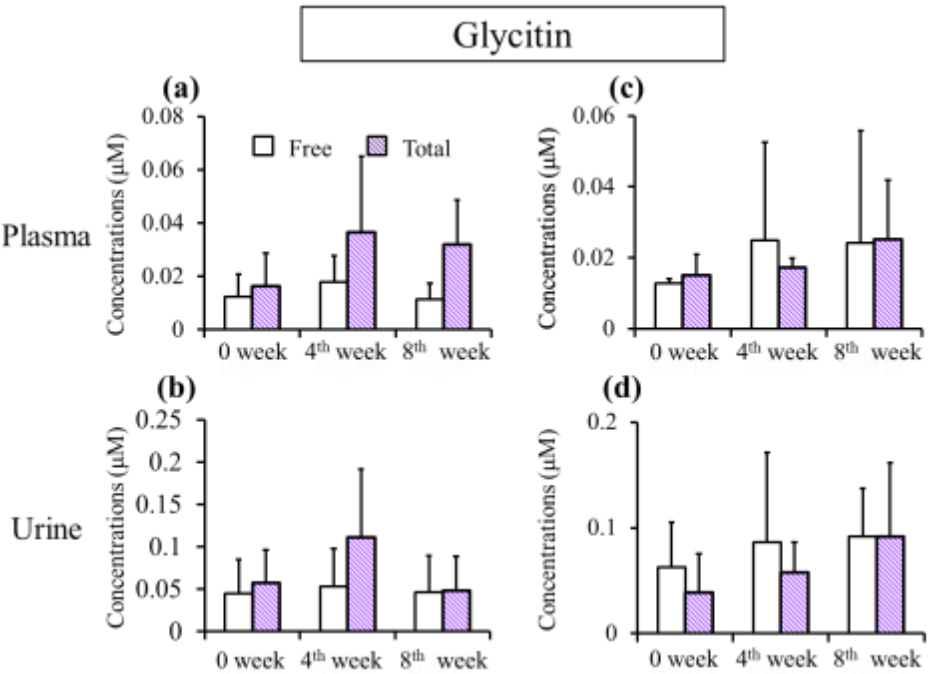


Figure 4. S1F. Concentrations of glycitin in the plasma and urine of volunteers whose vascular age did not change throughout the trial (a, b) and volunteers whose vascular age slightly increased during the trial (c, d). Results are represented as means±SE.

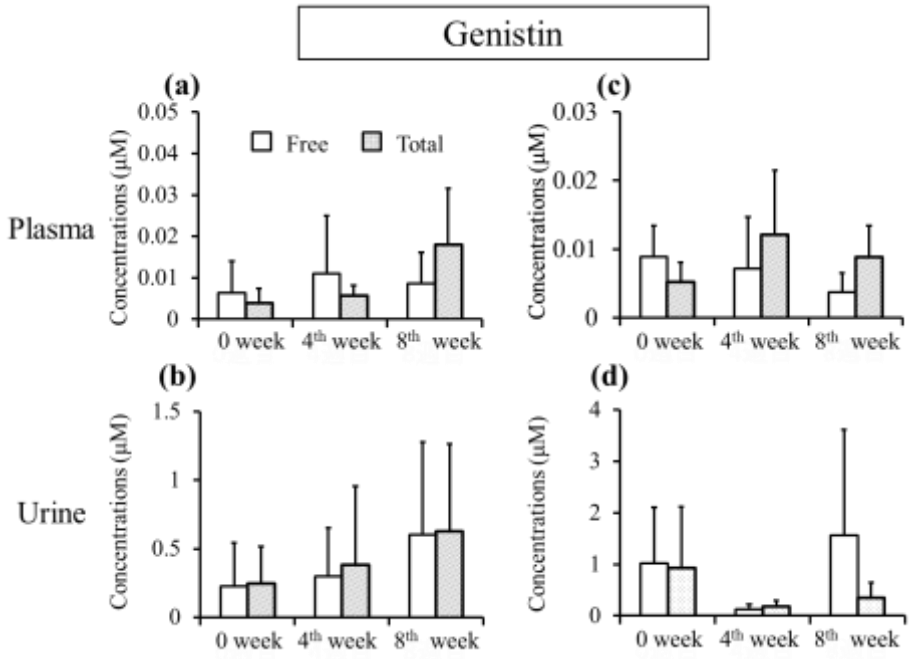


Figure 4. S1G. Concentrations of genistin in the plasma and urine of volunteers whose vascular age did not change throughout the trial (a, b) and volunteers whose vascular age slightly increased during the trial (c, d). Results are represented as means±SE.

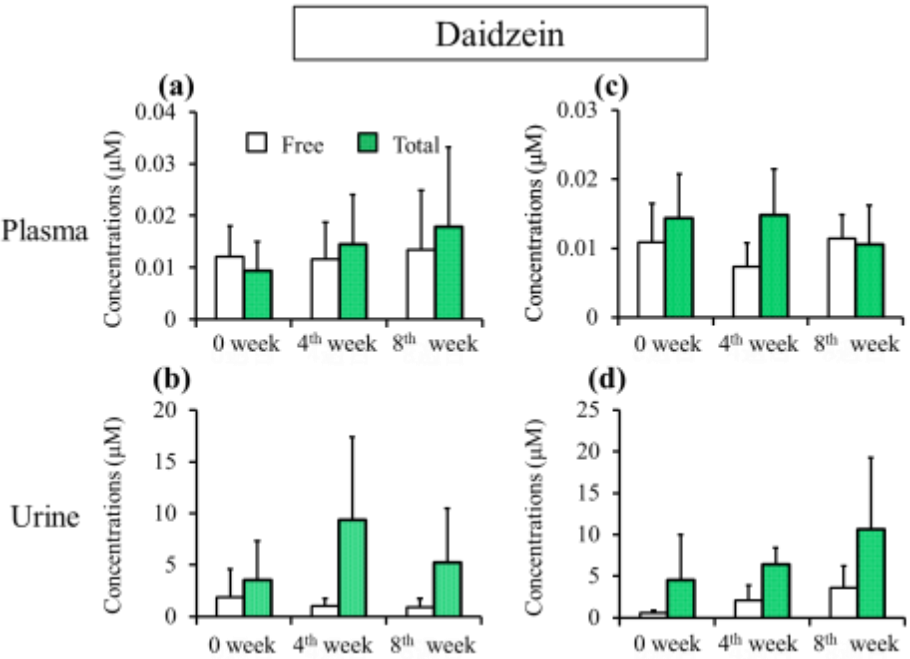


Figure 4. S1H. Concentrations of daidzein in the plasma and urine of volunteers whose vascular age did not change throughout the trial (a, b) and volunteers whose vascular age slightly increased during the trial (c, d). Results are represented as means±SE.

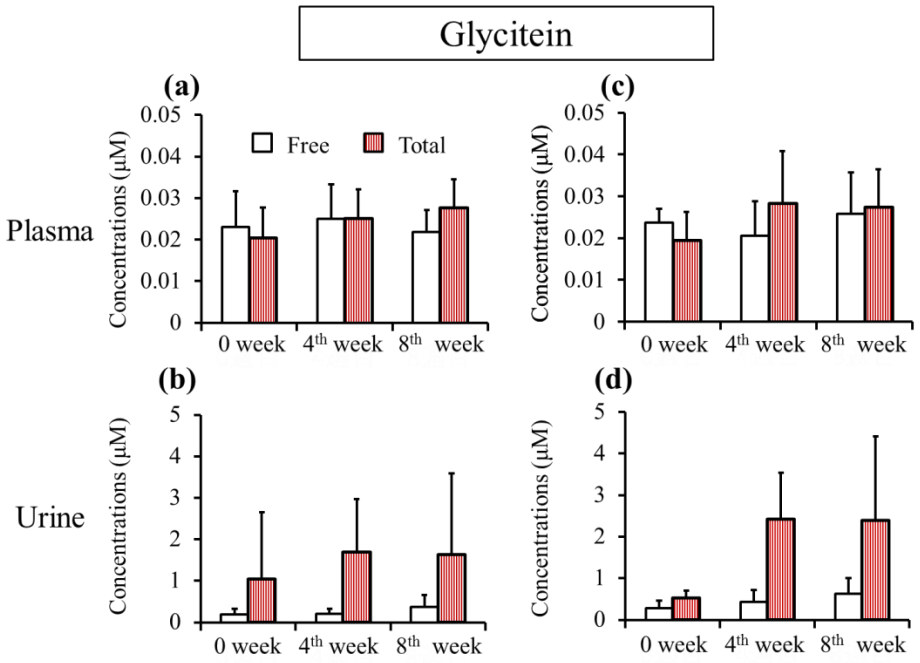


Figure 4. S1I. Concentrations of glycitein in the plasma and urine of volunteers whose vascular age did not change throughout the trial (a, b) and volunteers whose vascular age slightly increased during the trial (c, d). Results are represented as means±SE.

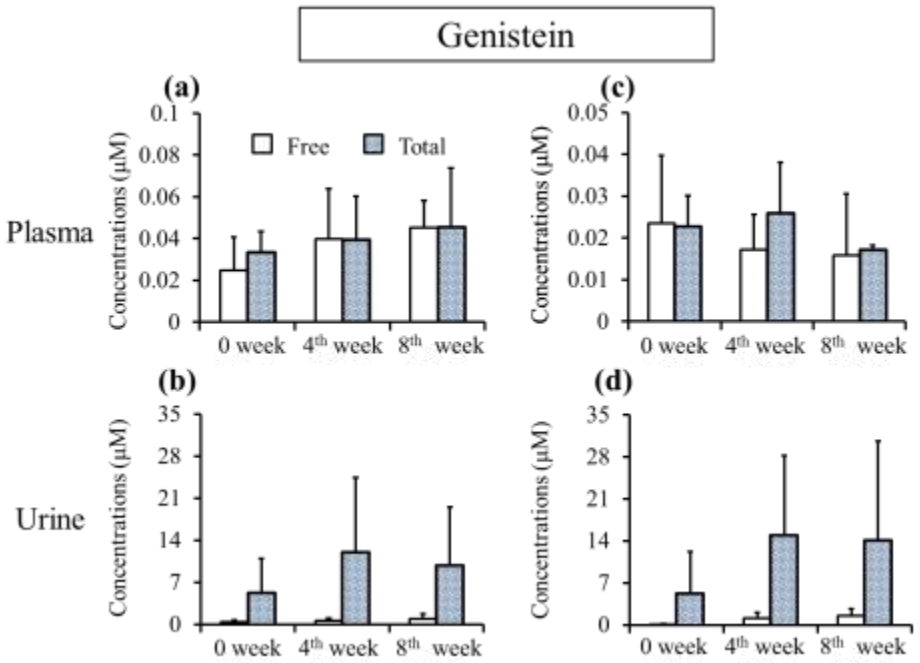


Figure 4. S1J. Concentrations of genistein in the plasma and urine of volunteers whose vascular age did not change throughout the trial (a, b) and volunteers whose vascular age slightly increased during the trial (c, d). Results are represented as means±SE.

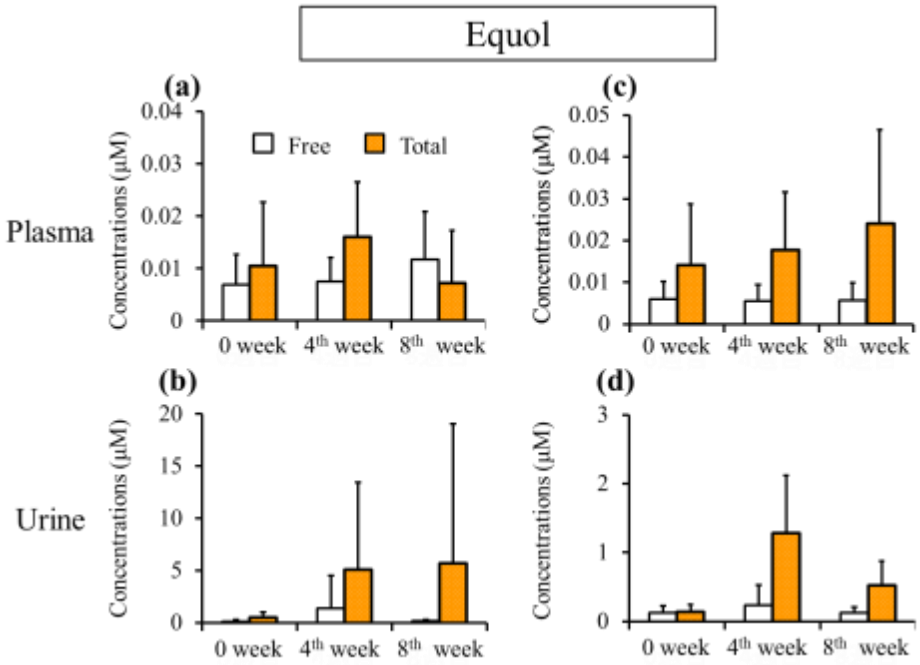


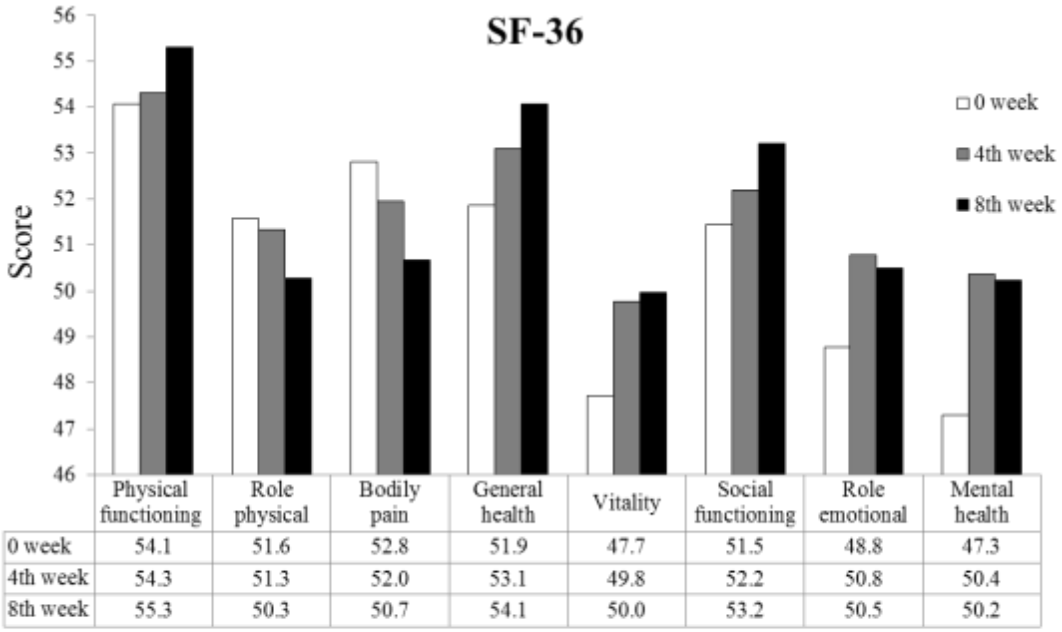
Figure 4. S1K. Concentrations of equol in the plasma and urine of volunteers whose vascular age did not change throughout the trial (a, b) and volunteers whose vascular age slightly increased during the trial (c, d). Results are represented as means±SE.

Figure 4. S2A. An association heat map between biomarkers and polyphenols in the plasma of volunteers from 'Improved at the 4th week' group. Each square indicates the Pearson correlation coefficient vales of corresponding factors. Positive correlations were shown in blue, negative ones were shown in red.

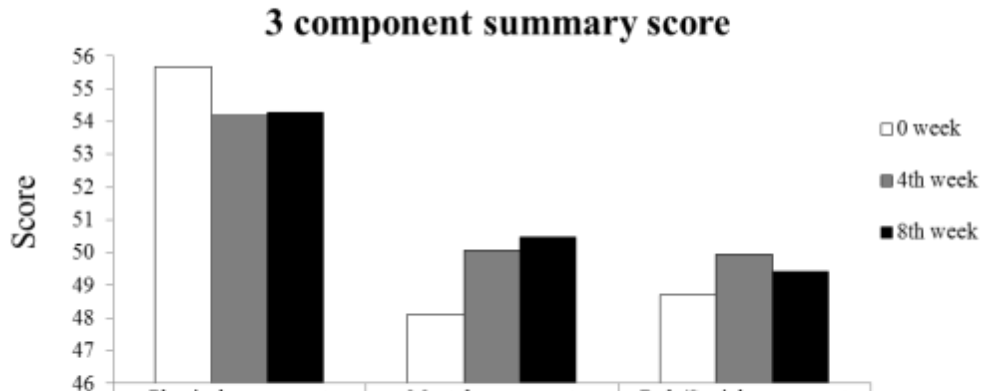
Figure 4. S2B. An association heat map between biomarkers and polyphenols in the urine of volunteers from 'Improved at the 4th week' group. Each square indicates the Pearson correlation coefficient vales of corresponding factors. Positive correlations were shown in blue, negative ones were shown in red.

Figure 4. S2C. An association heat map between biomarkers and polyphenols in the urine of volunteers from 'Improved at the 4th week' group. Each square indicates the Pearson correlation coefficient vales of corresponding factors. Positive correlations were shown in blue, negative ones were shown in red.

Figure 4. S2D. An association heat map between biomarkers and polyphenols in the urine of volunteers from 'Improved at the 4th week' group. Each square indicates the Pearson correlation coefficient vales of corresponding factors. Positive correlations were shown in blue, negative ones were shown in red.



	Physical functioning	Role physical	Bodily pain	General health	Vitality	Social functioning	Role emotional	Mental health
0 week	54.1	51.6	52.8	51.9	47.7	51.5	48.8	47.3
4th week	54.3	51.3	52.0	53.1	49.8	52.2	50.8	50.4
8th week	55.3	50.3	50.7	54.1	50.0	53.2	50.5	50.2



	Physical component summary	Mental component summary	Role/Social component summary
0 week	55.7	48.1	48.7
4th week	54.2	50.1	50.0
8th week	54.3	50.5	49.4

Figure 4. S2. HRQOL of volunteers before and after consumptionof roasted black soybeans.

Chapter 5. General discussion

Decline in the vascular function caused by aging and oxidative stress is the first step to CVD [4]. Preventive effect and improvement of polyphenol-rich food materials on vascular function with alteration of the oxidative stress is reported by many previous studies [10-13]. Black soybean (*Glycine max*) has been reported various health beneficaila functions, and it shows the greatest antioxidant efficiency compared to the other colored soybeans, owning to the high content of polyphenolic flavonoids including anthocyanidins and flavan-3-ols in its seed coat [17-20]. Moreover, black soybean also contains isoflavones in its cotyledons since it belongs to the soybean family. These polyphenols also contribute to anti-diabetes, anti-obesity, anti-hypolipidemia, anti-hyperglycemia, preventing oxidative stress and anti-inflammation [26, 40, 43, 66, 99, 118, 119]. However, the not fully understood bioavailability of black soybean polyphenols, their direct correlation with the beneficial effects, and a lack in evidence of its effects by human trial are the most challenging issues for applying black soybean as a functional food that exerts beneficial effects on the vascular function. This dissertation aimed to investigate the absorption, metabolism, distribution and excretion of black soybean flavan-3-ols using animal study with a proper analytical method to predict the possible bioavailability in human, and explore the effects of black soybean consumption on vascular function using human study.

To investigate the absorption, metabolism, distribution and excretion of orally consumed polyphenols, the analytical method used for quantifying the polyphenols is important. The author developed an analytical method using HPLC for the measurements of black soybean polyphenols (Chapter 2). Major polyphenol subclasses in the black soybean are anthocyanidins and flavan-3-ols in the seed coat [21-25], and isoflavones in the cotyledons [16]. The measurements of these polyphenols in the food material are usually achieved using HPLC by UV detection, ie. anthocyanidins were detected at 520 nm [120, 121]; flavan-3-ols were detected at 280 nm [91]; isoflavones

were detected at 254 nm [122]. Considering that the detection ranges of isoflavones and flavan-3-ols (254 nm and 280 nm) are too close, the fluorescence detection of flavan-3-ols was applied instead of UV detection. This HPLC system significantly improved the detection of flavan-3-ols by using fluorescence detection (Table 2.1), and all the polyphenols that were investigated in this dissertation were well separated without overlapping (Figure 4.3). Owing to that the separation of 4 flavan-3-ols and 7 isoflavones were achieved in one analysis by this method, much time and labor were saved. In addition to the proper instruments, extraction process of polyphneols in the biological samples was modified to minimize the loss of target compounds and the recovery was used to correct the 'real' concentrations (Table 2.4). However, the recovery of flavan-3-ols was affected by not only the type of the biological samples (ie. muscle, faeces, urine) but also the chemical structure of themselves (Figure Gd-1) due to the interaction between polyphenols and biological micro molecules [123]. Therefore, calibration curves of corresponding polyphenols in biological samples were used so that overestimating or underestimating the bioavailability of flavan-3-ols was avoided.

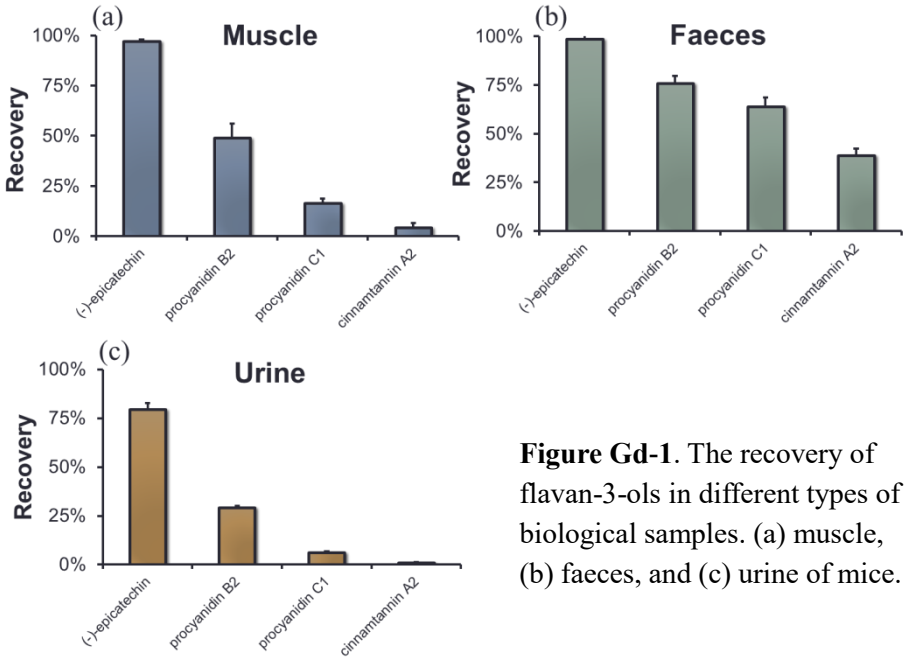


Figure Gd-1. The recovery of flavan-3-ols in different types of biological samples. (a) muscle, (b) faeces, and (c) urine of mice.

Using the developed method, further studies were conducted to investigate the absorption, metabolism, distribution and excretion of black soybean flavan-3-ols and explore the effects of black soybeans polyphenols on vascular function in human. In Chapter 3, (−)-epicatechin, procyanidin B2, procyanidin C1 and cinnamtannin A2, was investigated by administrating BE to ICR mice. Figure Gd-2 is the summarized

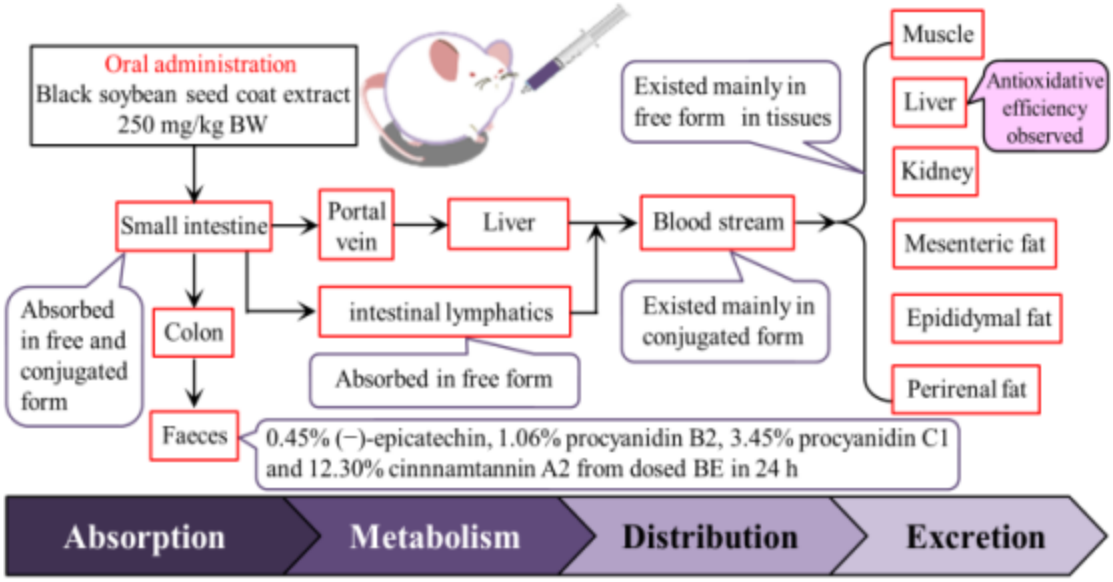


Figure Gd-2. Summarized illustration of oral bioavailability of black soybean flavan-3-ols reported in this dissertation.

illustration of flavan-3-ol bioavailability reported by this dissertation. Generally, polyphenols in aglycone form can be absorbed into the body [124]. Flavan-3-ols of black soybean are present in the aglycone form, but the polymer structures of them once made it hard to predict their possible bioavailability compared to other polyphenols. Recent studies suggested that like most polyphenols, flavan-3-ol had low oral bioavailability [37, 68, 74, 90, 91] and were not likely to exert the same antioxidative effect *in vivo* as reported by *in vitro* studies (effective concentrations were usually 1-10^2 μM). Meanwhile, results in this dissertation showed that flavan-3-ols were absorbed into the body and even at 10^{-1} μM or nmol/g tissue level (Figure 3.1C), significant

antioxidative efficiency *in vivo* was observed after the administration of BE (Figure 3.3). This evidence helps to explain the inconsistency between the bioavailability and beneficial effects of flavan-3-ols, and suggests that the consumption of black soybean may be an effective way to increase the polyphenol content in the body so that their reported beneficial effects can be achieved *in vivo*. (−)-Epicatechin, procyanidin B2, procyanidin C1 and cinnamtannin A2 are not the only flavan-3-ols in the BE. Pentamer and hexamer is also contained [25]. Since the authentic compounds are unavailable, the investigation of pentamer and hexamer is not conducted in this dissertation. Nevertheless, there is a possibility that detected (−)-epicatechin, procyanidin B2, procyanidin C1 and cinnamtannin A2 in the animal experiment (Chapter 3) may be the outcomes of broken procyanidins with higher DP than themselves, since that there is evidence showing pure procyanidin B2 breaks down into (−)-epicatechin in the upper digestive tract *in vivo* [37]. However, controversial results were reported by other study showed that procyanidins were not degraded into compounds with lower DP *in vivo* [83]. This inconsistency was also observed in cocoa procyanidins. Procyanidin oligomers (trimer to hexamer) which were purified from cocoa, decomposed to procyanidin monomers and dimers under acidic conditions that resembled the gastric environment *in vitro* [125]. In another study, cocoa procyanidins were stable during gastric transit in human after consumption of cocoa beverage [126].This inconsistency may be due to the difference between pure compounds and polyphenol-rich extracts. When procyanidins were administrated as pure compounds, they were easily affected by the acidic conditions, but when they were administrated with other components, the chance of them interacting with the acid was reduced, especially that procyanidin-rich extracts were usually dissolved into distilled water before administration which were supposed to increase the pH of the upper digestive tract. In this dissertation, flavan-3-ols were detected from small intestine 0.25 h after the oral administration of BE (Figure 3.1B) suggesting that the stay of BE in the stomach was too short for the procyandins to

decompose. These factors suggested that decomposition of procyanidins in this dissertation were unlikely to happen. In addition, a study showed that A-type (C4-C8 and C2-O-C7 linkage) procyanidin dimers were better absorbed than B-type (C4-C8 linkage) procyanidin dimer but the mechanism was not clear [74]. In this dissertation, the difference of bioavailability of A-type procyanidins and B-type procyanidins were not investigated since B-type procyandins were mainly contained in BE. Furthermore, procyanidins with C4-C6 linkage are also referred as B-type procyanidins, such as procyanidin B5, a procyanidin dimer with C4-C6 linkage, which was detected *in vivo* by both animal study and human study [127, 128], but the difference in the bioavailability of procyanidins with different linkages still remain unclear. Further studies using pure compounds or extracts with different composition of procyanidns from BE should be conducted to elucidate the bioavailability of all types of procyanidins. After all, (−)-epicatechin, procyanidin B2, procyanidin C1 and cinnamtannin A2 were absorbed *in vivo* and exerted oxidative efficiency after administration of BE.

Basing on the results of Chapter 3, possible effects of black soybean polyphenols on vascular function in human was investigated. The results showed that black soybean consumption improved vascular function (Table 4.3, Figure 4.1) and oxidative stress (Figure 4.2) by increasing the polyphenol content (Table 4.5-7) in the body of healthy human. The increased physiological concentrations of polyphenols after black soybean consumption was actually not surprising, but their significant correlation with the improved vascular function and oxidative stress (Figure 4.4) was strong evidence for black soybean consumption exerting health beneficial functions in human. The author also tried to find out the responsible component of black soybean polyphenols that contributed most to the observed health beneficial functions. Figure Gd-3 showed that the ratio of polyphenols from the seed coat of black soybeans (flavan-3-ols) was increased in the plasma of volunteers whose vascular age was lowered after black soybean consumption, while opposite change was observed in the volunteers whose

vascular age did not change or negatively changed during the trial. Similar change was not observed in the polyphenols from the cotyledons of black soybeans (isoflavones).

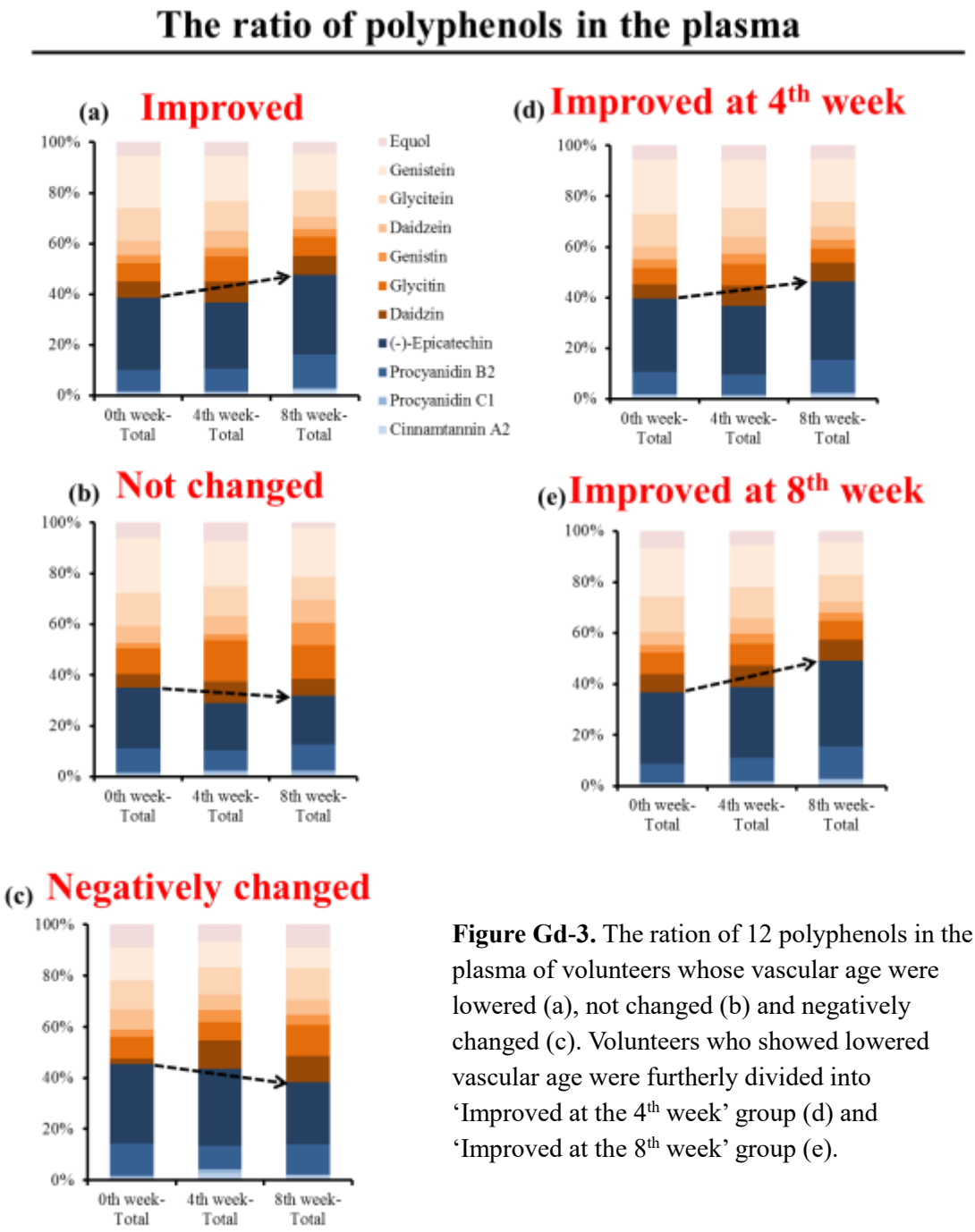


Figure Gd-3. The ration of 12 polyphenols in the plasma of volunteers whose vascular age were lowered (a), not changed (b) and negatively changed (c). Volunteers who showed lowered vascular age were furtherly divided into 'Improved at the 4th week' group (d) and 'Improved at the 8th week' group (e).

This result narrowed down the candidates of responsible component to black soybean flavan-3-ols. Using the association heat map (Figure 4.4), the candidates were furtherly narrowed down to 3 compounds, namely (−)-epicatechin, procyanidin B2 and

cinnamtannin A2. They were more closely correlated to the observed effects compared to procyanidin C1 and cyanidindin-3-*O*-glucoside, but the evidence was not strong enough to claim that they were the responsible components. Even though, results of this dissertation showed clearly that black soybean polyphenols (especially the flavan-3-ols) were bioactive *in vivo*, and they played an important role in the improvement of vascular function and oxidative stress in human after the consumption of black soybean. Although flavan-3-ols were detected both in the plasma of human and that of mice, flavan-3-ols in the plasma of human seemed to exist mainly in aglycone form (Table 4.5-7), while they existed mainly as their conjugated forms in the plasma of mice (Figure 3.1A, Table 3.1). This inconsistency is due to the difference of species in polyphenol metabolism. A previous study [80] showed that major metabolites of (−)-epicatechin in mice plasma were nonmethylated and methylated glucronides and sulfates. The total relative amount of nonmethylated conjugates reached 64.8%. In the meanwhile, the major metabolites of (−)-epicatechin in human plasma were more complicated than that in the mice plasma, and the total relative amount of nonmethylated conjugates were only 48.3%. In addition, 6 h after the consumption, the total relative amount of nonmethylated conjugates reduced to 11%, and the major metabolites were 5-carbon ring fission metabolites, such as 5-(4'-hydroxyphenyl)-γ-valerolactone-3'-sulfate and 5-(4'-hydroxyphenyl)-γ-valerolactone-3'-*O*-glucuronide. In this dissertation, β-glucuronidase and sulfatase were used to deconjugate the nonmethylated conjugates of polyphenols. Since the plasma of human was collect at least 8 h after the black soybean consumption, and the nonmethylated conjugates of flavan-3-ols were unlikely to exist as a great amount in the plasma at that time, detected flavan-3-ols did not show great difference after deconjugation by β-glucuronidase and sulfatase. By contrast, 8 h after administration, detected flavan-3-ols in the plasma of mice showed a clear increase after the deconjugation. Further detailed studies on the kinetic changes of procyanidins

in human are needed.

In addition to the intact compounds of black soybean polyphenols and their conjugates, the phenolic metabolites of polyphenols released from colonic metabolism [33, 35, 80-82] may also contribute to the observed beneficial effects. Equol, has a similar structure to estrogen, and binds to estrogen receptors to exhibit estrogenic activity [129]. It is also reported that equol attenuates atherosclerosis by inhibiting endoplasmic reticulum stress via activation of Nrf2 in endothelial cells [130], and equol producer-phenotype is associated with improvements in blood pressure arterial stiffness and endothelial function [131-133]. Evidence of their effects on human health and related bioavailability needs to be clarified, probably started with the development of a proper analytical method because of the complexity and large amount. Besides polyphenols, other components in the black soybean are also reported exerting health beneficial functions. Soy protein, accounting for approximately 36% of dry soybeans by weight, was reported to lower the risk of CVD by regulating the lipid profile, including lowering total cholesterol, low-density lipoprotein and triglycerides without affecting high-density lipoprotein [134]. A study even indicated that soy protein itself, not the isoflavones, reduced oxidative stress in rats [135]. Polyphenol-protein complex and its consequences for related health beneficial functions is another factor that should also be taken into consideration. *Artemisia drucunculus L.* is a perennial herb, and the polyphenols of it (bioactive compounds of which were identified as 6-demethoxycapillarisin, davidigenin, sakuranetin, 2',4-dihydroxy-4'-methoxydihydrochalcone, and 2',4'-dihydroxy-4-methoxydihydro-chalcone) complexed to soy protein showed enhanced hypoglycemic activity and bioavailability in mice compared to the treatment of the polyphenols only [136]. Therefore, black soybean is a nutritious food and a potential functional food to improving vascular function. To furtherly specifying the responsible content of the black soybean and its underlying mechanism is an important issue.

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List of publications

A. Original papers

Wang L, Yamashita Y, Saito A and Ashida H. An analysis method for flavan-3-ols using high performance liquid chromatography coupled with a fluorescence detector. *J Food Drug Anal.* 2017, **25**, 478-487.

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