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## Original Article

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

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## ABSTRACT

Procyanidins belong to a family of flavan-3-ols, which consist of monomers, (+)-catechin and (–)-epicatechin, and their oligomers and polymers, and are distributed in many plant-derived foods. Procyanidins are reported to have many beneficial physiological activities, such as antihypertensive and anticancer effects. However, the bioavailability of procyanidins is not well understood owing to a lack of convenient and high-sensitive analysis methods. The aim of this study was to develop an improved method for determining procyanidin content in both food materials and biological samples. High performance liquid chromatography (HPLC) coupled with a fluorescence detector was used in this study. The limits of detection (LODs) of (+)-catechin, (–)-epicatechin, procyanidin B2, procyanidin C1, and cinnamtannin A2 were  $3.0 \times 10^{-3}$  ng,  $4.0 \times 10^{-3}$  ng,  $14.0 \times 10^{-3}$  ng,  $18.5 \times 10^{-3}$  ng, and  $23.0 \times 10^{-3}$  ng, respectively; the limits of quantification (LOQs) were  $10.0 \times 10^{-3}$  ng,  $29.0 \times 10^{-3}$  ng,  $28.5 \times 10^{-3}$  ng,  $54.1 \times 10^{-3}$  ng, and  $115.0 \times 10^{-3}$  ng, respectively. The LOD and LOQ values indicated that the sensitivity of the fluorescence detector method was around 1000 times higher than that of conventional HPLC coupled with a UV-detector. We applied the developed method to measure procyanidins in black soybean seed coat extract (BE) prepared from soybeans grown under three different fertilization conditions, namely, conventional farming, basal manure application, and intertillage. The amount of flavan-3-ols in these BEs decreased in the order intertillage > basal manure application > conventional farming. Commercially available BE was orally administered to mice at a dose of 250 mg/kg body weight, and we measured the blood flavan-3-ol content. Data from plasma analysis indicated that up to the tetramer oligomerization, procyanidins were detectable and flavan-3-ols mainly existed in conjugated forms in the plasma. In conclusion, we developed a highly sensitive and convenient analytical method for the analysis of flavan-3-ols, and applied this technique to investigate the bioavailability of flavan-3-ols in biological samples and to measure flavan-3-ol content in food material and plants.

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## 1. Introduction

Flavan-3-ols are the most common group of flavonoids in plant-derived foods and beverages. Flavonoids have beneficial biological effects, including strong antioxidative [1], anticarcinogenic [2], antiobesity [3], and antidiabetic effects. We previously showed that oral administration of a procyanidin-rich cacao liquor extract facilitated glucose uptake and improved postprandial hyperglycemia by promoting glucose transporter 4 (GLUT4) translocation in muscle cells *in vitro* and *in vivo* [4]. Another group showed that oral administration of procyanidins from lotus seedpods significantly inhibited the growth of melanoma in C57BL/6J mice, and decreased tumor weight by approximately 55% [5].

For the measurement of procyanidins in plant extract, high performance liquid chromatography (HPLC) and HPLC/tandem mass spectrometry (LC-MS/MS) are good tools [6]. However, the bioavailability of procyanidins after ingestion is not well understood. Furthermore, in many previous studies, the specific chemical structures of procyanidins extracted from food materials have not been well characterized. Procyanidin-rich extracts contain procyanidin dimers, trimers, and tetramers, such as procyanidin B2, procyanidin C1, and cinnamtannin A2. Moreover, the analytical method used greatly affects the limit of determination of procyanidins in biological samples. In a study performed by intragastric gavage of 1 g/kg body weight grape seed procyanidin extract to Wistar rats, procyanidin dimers and trimers in the aglycone form were detected by LC-MS/MS. These dimers and trimers reached maximum concentrations in the plasma of 0.57 nM and 0.03 nM, respectively, 1 hour after ingestion [7]. In another study using the same dose and animals with a different extraction method, the concentrations of dimers and trimers were reported as 2.4  $\mu$ M and 8.55  $\mu$ M, respectively, 2 hours after ingestion [8]. The proportion of total procyanidins recovered from the samples was not considered in either case. Therefore, a validated analytical method with proper evaluation is needed for the detection of procyanidins in biological samples.

Owing to the complexity of their chemical structures, procyanidins are difficult to fully characterize. Chromatographic separation of procyanidins using HPLC is widely used and has become a standard analysis method [9]. HPLC instruments are normally equipped with UV detectors; however, UV detectors are not sensitive enough for the detection of procyanidins at the very low concentrations that are common in biological samples. Many practical separation methods have been reported for separating procyanidins based on their molecular weight or hydrophobicity [10–12]. These methods can achieve separation of procyanidin oligomers and polymers from food extracts. Often these methods are coupled with mass spectrometry to identify procyanidins from their fragmentation patterns [13]. The main disadvantage of these methods is that the fragmentation patterns of procyanidin compounds show no differences among isomers having the same degree of polymerization. Having authentic compounds may improve the ability to discern between isomers;

however, mass spectrometry remains an expensive technique. Fluorescence detection of procyanidins has the potential benefits of high sensitivity and the ability to reduce interfering signals [13]. Considering the budget, HPLC coupled with a fluorescence detector should be a better choice compared to mass spectrometry. Therefore, in the present study we developed a highly sensitive and practical analysis method for procyanidins using HPLC coupled with a fluorescence detector (HPLC-FLD).

## 2. Material and methods

### 2.1. Materials

(+)-Catechin ( $\geq 98.9\%$ ) and (–)-epicatechin ( $\geq 98.9\%$ ) were purchased from Kurita Analysis Service Co. Ltd. (Ibaraki, Japan). Authentic compounds of procyanidin B2, procyanidin C1, cinnamtannin A2, and ChronoCare, a commercially available black soybean seed coat extract (BE), were obtained from Fujicco Co., Ltd. (Kobe, Japan). Procyanidin B3-OAc, used as internal standard, was synthesized and purified by solid phase extraction [14]. HPLC grade methanol, acetonitrile, formic acid, and phenol reagents for the Folin–Ciocalteu method were obtained from Wako Pure Chemical Industries (Osaka, Japan).  $\beta$ -Glucuronidase from *Escherichia coli* (Type IX-A) was purchased from Sigma-Aldrich Co. LLC (St. Louis, MO, USA). All other reagents and chemicals were of analytical grade, unless otherwise stated.

### 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 an LC work station (Shimadzu Corporation, Kyoto, Japan). A Cadenza CL-C18 column ( $\phi$  250 mm  $\times$  4.6 mm, 3  $\mu$ m, Imtakt, Kyoto, Japan) was selected and a guard column (Cadenza CL-C18,  $\phi$  5 mm  $\times$  2 mm, 3  $\mu$ m, Imtakt) was used to protect the analytical column.

### 2.3. Chromatographic conditions

All samples were analyzed using the HPLC system described in the equipment section, with 0.1% formic acid solution as mobile phase A and acetonitrile as mobile phase B. Separation of procyanidins was achieved using a linear gradient from 5–15% B over 0–45 minutes; 15–80% B over 45–50 minutes; 80% B over 50–53 minutes; and 5% B over 53–70 minutes. The gradient from 53 minutes onwards was set for re-equilibration between samples. The flow rate was set at 0.7 mL/min. The injection volume was 10  $\mu$ L and the temperature of the column oven was 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 to allow a comparison of the different methods.

## 2.4. Limit of detection and limit of quantification

In this study, all authentic compounds (Figure 1) were dissolved in 50% methanol to form 1mM stock solutions and stored at  $-20^{\circ}\text{C}$  until analysis. Stock solutions were diluted to a series of different concentrations for detection. 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).

## 2.5. Calibration curves

A series of standard solutions of (+)-catechin, (–)-epicatechin, procyanidin B2, procyanidin C1, and cinnamtannin A2 across a concentrations range of  $0.001\mu\text{M}$  to  $10\mu\text{M}$  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.

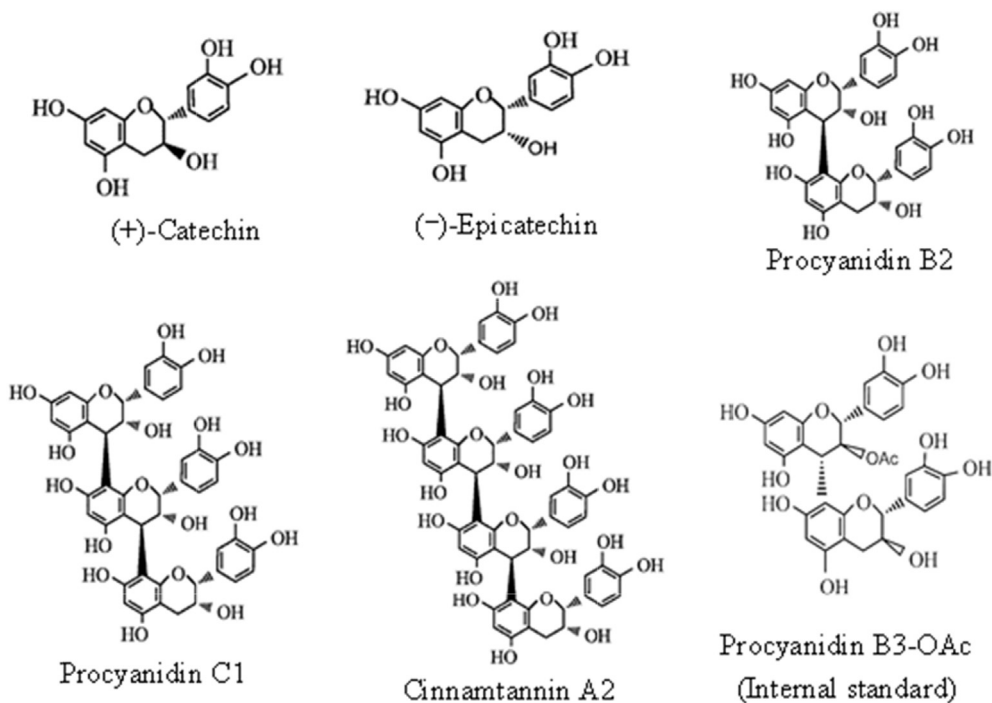
## 2.6. Preparation of BE

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 (Tajima-Dori, Ltd., Toyooka, Japan), 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, which was applied to the soil at  $0.06\text{ kg/m}^2$  before seeding. Intertillage also involved cultivation with the same fermented organic fertilizer ( $0.06\text{ kg/m}^2$ ), which was applied to the soil during the growth of the black soybeans. For analysis of the procyanidin content of the BEs, extraction was performed according to a previous report by Ito et al [15] 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) three 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 redissolved in 50% methanol to a concentration of 30 mg/mL to prepare the stock solutions, which were preserved at  $-20^{\circ}\text{C}$  until analysis.

## 2.7. Analysis of BE

To estimate the content of (+)-catechin, (–)-epicatechin, procyanidin B2, procyanidin C1, and cinnamtannin A2 in the BE, a stock solution of each authentic compound was diluted to a concentration of 0.1 mg/mL and analyzed by HPLC. 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 [16] with some



**Figure 1** – Chemical structures of flavan-3-ols in BE. (+)-Catechin, (–)-epicatechin, procyanidin B2, procyanidin C1, and cinnamtannin A2 were the main flavan-3-ols in BE. Procyanidin B3-OAc was used as an internal standard for quantitative analysis of procyanidins and was not detected in BE. BE = black soybean seed coat extract.

modifications. Briefly, 0.5 mL of the 0.1 mg/mL BE solution was added to 2.5 mL of 0.2N phenol reagent and mixed in a glass test tube. After incubation for exactly 3 minutes, 2.5 mL of 10% (w/v)  $\text{Na}_2\text{CO}_3$  solution was added to terminate the reaction. After 1 hour, the absorbance was measured at 750 nm. To make a calibration curve, gallic acid was used as a standard compound over a concentration range of 0.025–0.1 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 the oxygen radical absorbance capacity method (ORAC) [17]. All reagents were dissolved and diluted in 75mM potassium phosphate buffer (pH 7.4). A 20- $\mu\text{L}$  aliquot of diluted sample was mixed with 120  $\mu\text{L}$  of 70nM fluorescein and added to a 96-well plate. After incubation at 37°C for 15 minutes, 60  $\mu\text{L}$  of 12mM 2,2'-azobis-2-methylpropanimidamide dihydrochloride was quickly added to the mixture for detection. The total volume of the reaction mixture was miniaturized to 200  $\mu\text{L}$ . To make a calibration curve, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (trolox) was used as a standard compound over a concentration range of 0–1mM. The fluorescence intensity was measured every 2 minutes over 90 minutes by a Wallac Multilabel Counter (ARVO SX; PerkinElmer, Boston, MA, USA) at excitation and emission wavelengths of 485 nm and 535 nm, respectively. The oxidant capacity of the BE was measured and expressed as  $\mu\text{mol}$  trolox equivalent/g BE according to the calibration curve.

## 2.8. Animal experiment

### 2.8.1. Experimental design

Animal experiments in this study were approved by our 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  $22 \pm 3^\circ\text{C}$  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, Japan SLC). Prior to the experiments, mice were acclimatized for 1 week. After acclimatization, ICR mice were randomly divided into 2 groups: control ( $n = 3$ ) and BE ( $n = 6$ ). The BE group were orally administered ChronoCare at a dose of 250 mg/kg body weight after 8 hours fasting, while the control group were given the same volume (200  $\mu\text{L}$ ) of distilled water as a vehicle. The dose of ChronoCare was decided from our previous animal study using procyanidin-rich cacao extract [4]. One hour after administration, the mice were sacrificed by drawing blood from cardiac puncture under anesthesia using pentobarbital. Plasma was isolated from the blood by centrifugation at 3000g for 10 minutes at 4°C and stored immediately at  $-80^\circ\text{C}$  until analysis.

### 2.8.2. Extraction efficiency and plasma analysis

To evaluate the loss during the extraction process, extraction efficiency 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  $\mu\text{L}$  plasma mixed with 10  $\mu\text{L}$  of 1mM ascorbic acid, which was added to prevent oxidation of procyanidins during extraction, was added to a

PFA tube (15 mL; Savillex, Minnetonka, MN, USA).  $\beta$ -Glucuronidase (500 U/sample) was used for deconjugation. After 1 hour incubation at 37°C, 1 mL of acetonitrile was added to the mixture to terminate the reaction and extract the procyanidins. The mixture was sonicated for 5 minutes and centrifuged at 3000g for 10 minutes. The supernatant was collected, and the extraction step was repeated two more times. Pooled supernatant was evaporated to dryness under a nitrogen stream in a fume hood. Dried material was dissolved in 50  $\mu\text{L}$  of 50% methanol and applied to HPLC.

## 2.9. Statistical analysis

Data are expressed the mean  $\pm$  standard deviation. Statistical analysis of data was performed by Dunnett's test using JMP statistical software version 11.2.0 (SAS Institute, Cary, NC, USA), and the level of significance was set at  $p < 0.05$ .

## 3. Results and discussion

### 3.1. Method validation

Standard compounds were analyzed by HPLC to identify procyanidins. The retention times of (+)-catechin, (–)-epicatechin, procyanidin B2, procyanidin C1, and cinnamtannin A2 were 20.4 minutes, 28.9 minutes, 24.9 minutes, 34.3 minutes, and 37.7 minutes, respectively. Procyanidin B1 and (–)-epicatechin-(4 $\beta$ →6)-(–)-epicatechin-(4 $\beta$ →8)-(–)-epicatechin-(4 $\beta$ →8)-(–)-epicatechin (referred to as procyanidin 4-1 in this experiment), which are isomers of procyanidin B2 and cinnamtannin A2, respectively, were also analyzed. These compounds were well separated and showed no overlap with any other procyanidin compounds analyzed in this study, including their isomers (Figure 2). Because the major procyanidin oligomers detected in BE were procyanidin B2, procyanidin C1, and cinnamtannin A2, other compounds, such as procyanidin B1, were not measured quantitatively in this study.

The LOD and LOQ of procyanidins were measured by HPLC coupled with a UV detector (HPLC-UV) and HPLC-FLD. As shown in Table 1, the LODs of (+)-catechin, (–)-epicatechin, procyanidin B2, procyanidin C1, and cinnamtannin A2 detected by HPLC-UV were 1.7 ng, 2.9 ng, 25.0 ng, 40.0 ng, and 30.0 ng, respectively, where the LOD values for these compounds detected by HPLC-FLD were  $3.0 \times 10^{-3}$  ng,  $4.0 \times 10^{-3}$  ng,  $14.0 \times 10^{-3}$  ng,  $18.5 \times 10^{-3}$  ng, and  $23.0 \times 10^{-3}$  ng, respectively. The LOQs of (+)-catechin, (–)-epicatechin, procyanidin B2, procyanidin C1, and cinnamtannin A2 detected by HPLC-UV were 7.7 ng, 14.5 ng, 40.0 ng, 150.0 ng, and 500.0 ng, respectively, and the LOQs detected by HPLC-FLD were  $10.0 \times 10^{-3}$  ng,  $29.0 \times 10^{-3}$  ng,  $28.5 \times 10^{-3}$  ng,  $54.1 \times 10^{-3}$  ng, and  $115.0 \times 10^{-3}$  ng, respectively. The correlation coefficients ( $R^2$ ) of all calibration curves were  $\geq 0.999$ , and showed good linearity, allowing for quantitative analysis (Table 2). Many chromatographic separations used for the determination of procyanidins are based on HPLC-UV; however, we doubted their effectiveness because of the inherent low sensitivity and specificity of UV detection. We used a fluorescence detector to optimize the detection of



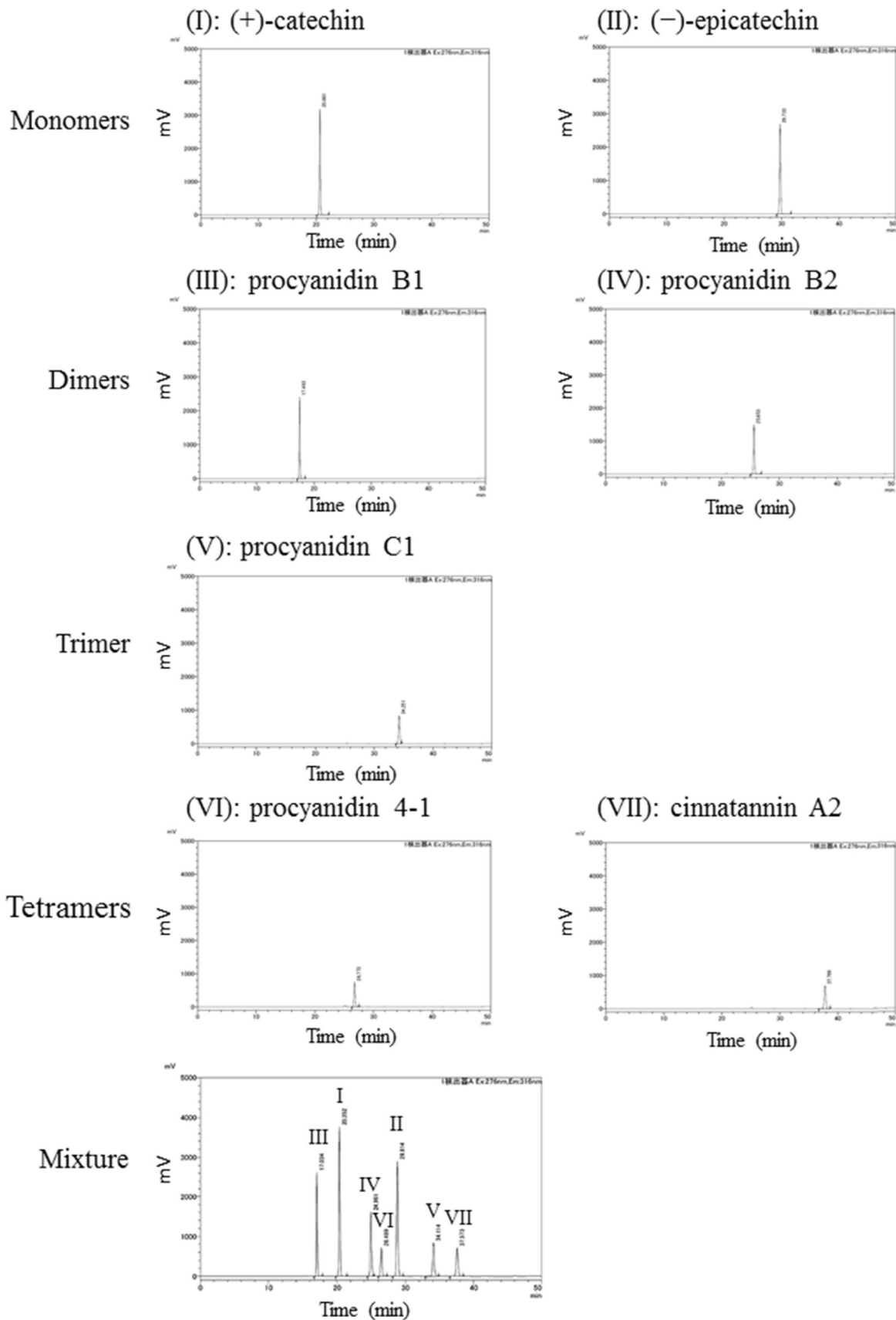


Figure 2 – Typical chromatogram of flavan-3-ols. Peak identification: (I) (+)-catechin, (II) (-)-epicatechin, (III) procyanidin B1, (IV) procyanidin B2, (V) procyanidin C1, (VI) procyanidin 4-1, (VII) cinnatannin A2.

**Table 1 – Limit of detection (LOD) and limit of quantification (LOQ) of flavan-3-ols detected by UV and fluorescence (-FLD) detectors.**

Compound	LOD <sup>a</sup> (ng)		LOQ <sup>b</sup> (ng)	
	HPLC-UV <sup>c</sup>	HPLC-FLD <sup>d</sup>	HPLC-UV <sup>c</sup>	HPLC-FLD <sup>d</sup>
(+)-Catechin	1.7	$3.0 \times 10^{-3}$	7.7	$10.0 \times 10^{-3}$
(-)-Epicatechin	2.9	$4.0 \times 10^{-3}$	14.5	$29.0 \times 10^{-3}$
Procyanidin B2	25.0	$14.0 \times 10^{-3}$	40.0	$28.5 \times 10^{-3}$
Procyanidin C1	40.0	$18.5 \times 10^{-3}$	150.0	$54.1 \times 10^{-3}$
Cinnamtannin A2	30.0	$23.0 \times 10^{-3}$	500.0	$115.0 \times 10^{-3}$

HPLC = high performance liquid chromatography.  
<sup>a</sup> LOD is defined as the absolute amount of analyte when its signal-to-noise ratio is 3.  
<sup>b</sup> LOQ is defined as the absolute amount of analyte when its signal-to-noise ratio is 10.  
<sup>c</sup> Absorbance monitored at 280 nm.  
<sup>d</sup> Excitation at 276 nm and emission monitored at 316 nm.

procyanidins. The fluorescence properties of procyanidins were first reported by Cho et al [18], and their peak excitation and emission were determined to be around 280 nm and 321–324 nm, respectively. These properties have been used to analyze procyanidin in white wines by Carando et al [19]. These methods were suitable for analyzing procyanidins from food material, but not for rigorous analysis of procyanidin bioavailability. The LODs and LOQs measured in this study using HPLC-FLD were approximately 1000-fold lower than those achievable using HPLC-UV. Fluorescence detection offers much higher sensitivity than UV detection and is a better choice for the detection of procyanidin oligomers. Considering the cost of expendables and routine maintenance of mass spectrometry, the HPLC-FLD method developed in this study can address similar research questions on a limited budget.

### 3.2. Application of HPLC coupled with a fluorescence detector

#### 3.2.1. Procyanidin contents and BE from black soybeans grown under three different conditions

HPLC-FLD was applied to analyze the procyanidin contents in BE (Figure 3). For soybeans grown by conventional farming, the BE contents of (+)-catechin, (-)-epicatechin, procyanidin B2, procyanidin C1, and cinnamtannin A4 were 0.17 mg/g BE, 21.34 mg/g BE, 10.55 mg/g BE, 7.31 mg/g BE, and 5.95 mg/g BE,

respectively. For the BE from soybeans grown with basal manure application, the BE contents of (+)-catechin, (-)-epicatechin, procyanidin B2, procyanidin C1, and cinnamtannin A4 were 0.20 mg/g BE, 22.84 mg/g BE, 11.62 mg/g BE, 8.50 mg/g BE, and 7.32 mg/g BE, respectively, and the BE of soybeans grown under intertillage conditions had contents of 0.22 mg/g BE, 24.63 mg/g BE, 12.44 mg/g BE, 8.83 mg/g BE, and 7.51 mg/g BE, respectively. The amount of flavan-3-ols from the three BEs decreased in the order intertillage > basal manure application > conventional farming (Figure 4). The total polyphenol contents and antioxidant capacity measured by the Folin–Ciocalteu method and ORAC also followed a similar trend (Figure 5). 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.

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 owing to higher polyphenol contents [20–23]. According to the results of this study, the polyphenol content, including procyanidins from black soybean seed coats, may yield a higher antioxidant capacity when organic farming methods are used. To the best of our knowledge, this is the first demonstration of procyanidin content in BE being influenced by fertilization conditions. Further study is required to clarify the cause of the high procyanidin content in BE from soybeans grown with intertillage.

#### 3.2.2. Concentrations of procyanidins in plasma of mice given ChronoCare

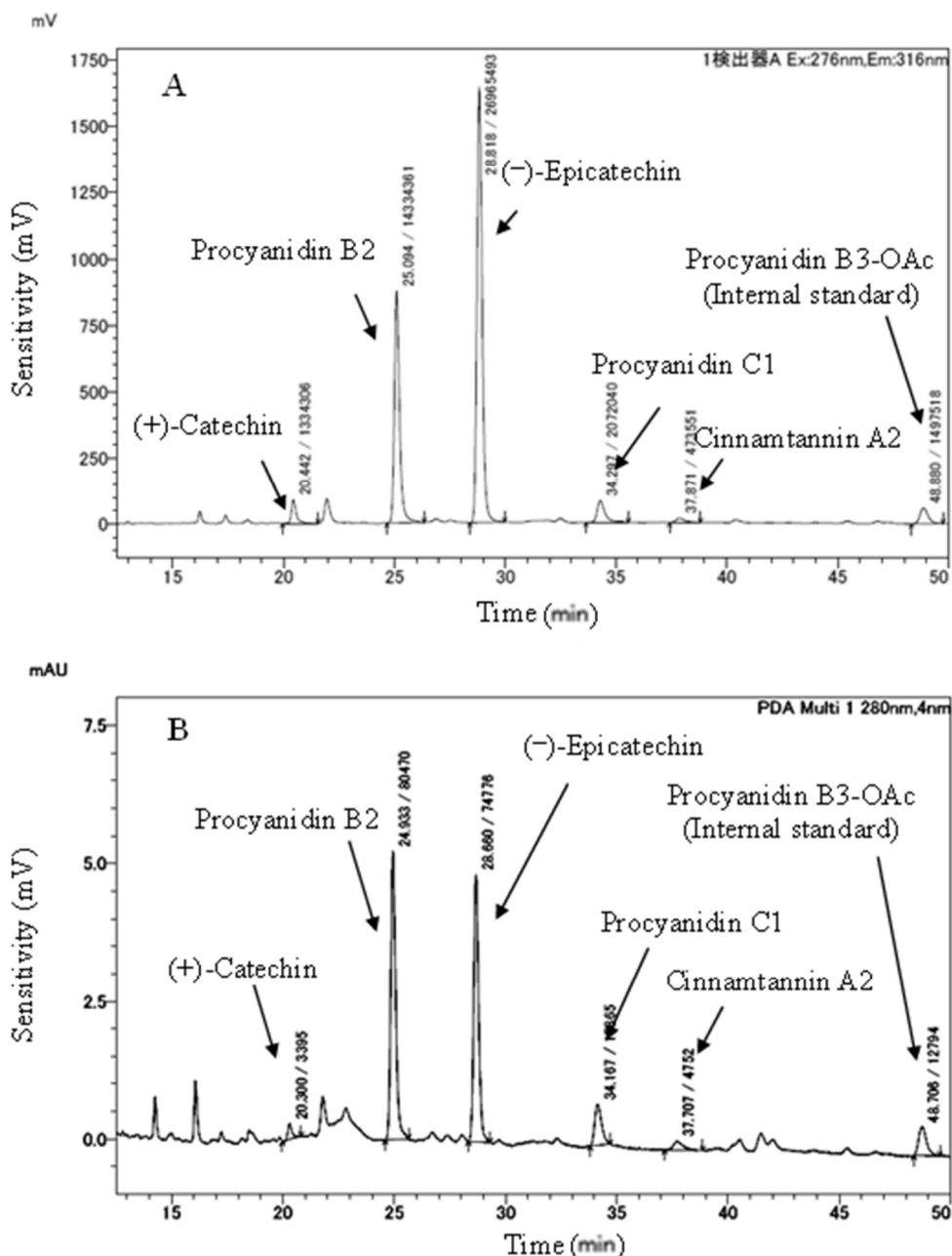
A preliminary experiment to elucidate procyanidin bioavailability was conducted. ChronoCare was orally administered to mice as a source of mixed procyanidins. The polyphenol composition and the extraction efficiency are shown in Tables 3 and 4, respectively. After deconjugation, (+)-catechin, (-)-epicatechin, procyanidin B2, procyanidin C1, and cinnamtannin A2 were detected and quantified as the total amount of corresponding aglycone plus conjugate forms. One hour after administration, the concentration of (-)-epicatechin aglycone in the plasma was 0.75 μM, and the concentration of its aglycone plus its conjugated forms was 20.11 μM. (+)-Catechin, procyanidin B2, procyanidin C1, and cinnamtannin A2 aglycone were only detected in trace amounts and could not be quantified. The concentration of

**Table 2 – Analytical parameters of flavan-3-ols by HPLC with fluorescence detector.**

Compound	Retention time (min)	R <sup>2</sup>	Linearity (μM)	Calibration curve <sup>a</sup>
(+)-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

HPLC = high performance liquid chromatography.

<sup>a</sup> Where x and y are the concentration of each compound and the ratio of the peak area of the compound to that of the internal standard, respectively.

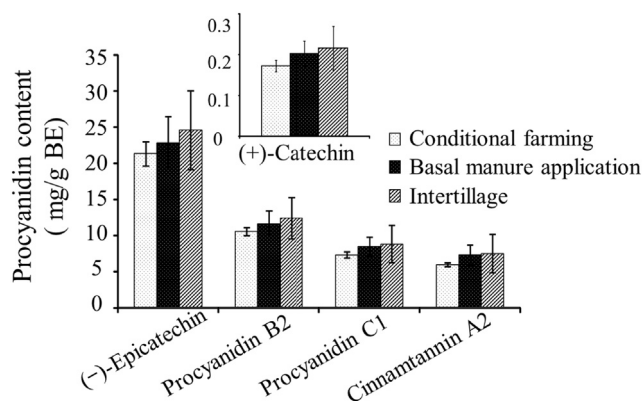


**Figure 3** – Typical chromatogram of 0.1 mg/mL BE. Flavan-3-ols were detected by (A) a fluorescence detector and (B) a UV detector. BE = black soybean seed coat extract.

(+)-catechin aglycone plus its conjugated forms was  $0.29\mu\text{M}$ , and those of procyanidin B2, procyanidin C1, and cinnamtannin A2 were  $0.07\mu\text{M}$ ,  $0.02\mu\text{M}$ , and  $0.01\mu\text{M}$ , respectively (Figure 6). (-)-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. The analytical method used greatly affected the LOD of procyanidins in biological samples. Considering the relatively low recovery of cinnamtannin A2 (68.7%), the total amount of cinnamtannin A2 aglycone and its conjugated forms should have been

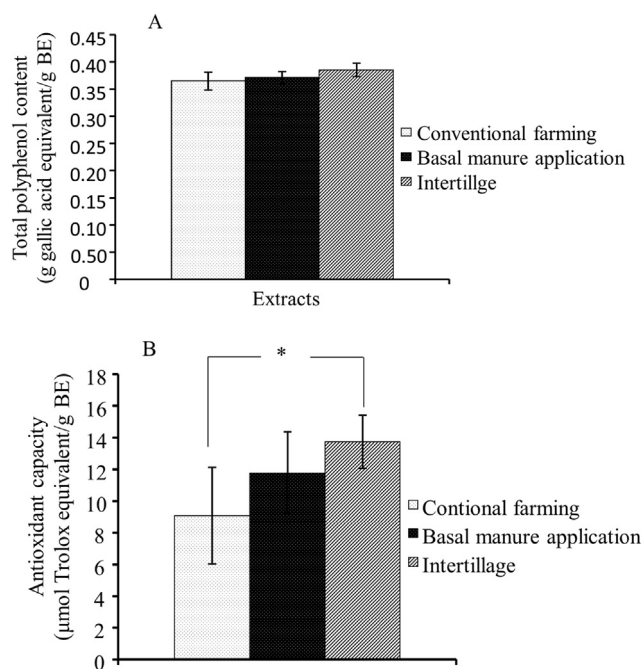
$0.015\mu\text{M}$ . However, the tetramer procyanidin was still present in the lowest amounts among the procyanidins. The trace detection of procyanidin B2, procyanidin C1, and cinnamtannin A2 aglycone was considered to be owing to several reasons, such as their low bioavailability, degradation of their oligomers, and the low administration dose and/or sampling volume. A study on the bioavailability of a procyanidin dimer performed by Baba et al [24] showed that 30 minutes after oral administration of procyanidin B2 to rats at a dose of 50 mg/kg body weight, the concentration of procyanidin B2 detected in the plasma was  $0.5\mu\text{M}$ . In the same study, a portion of





**Figure 4 – Content of flavan-3-ols in BE of black soybeans cultivated by three different fertilization methods. The amounts of flavan-3-ols were measured using HPLC-FLD and are represented as mg/g BE. BE = black soybean seed coat extract; HPLC-FLD = high performance liquid chromatography coupled with a fluorescence detector.**

procyanidin B2 degraded to (-)-epicatechin and the metabolized conjugated and/or methylated (-)-epicatechin were also observed. Because relatively high concentrations of (-)-epicatechin conjugates were also observed in this study,



**Figure 5 – Total polyphenol content and antioxidant capacity in the BE of black soybeans cultivated by three different fertilization methods. (A) Total polyphenol content was measured by the Folin–Ciocalteu method, and is represented as g gallic acid equivalent/g BE. (B) Antioxidant capacity was measured by ORAC and is represented as μmol Trolox equivalent/g BE. \* Significant difference from conventional farming by Dunnett's test. BE = black soybean seed coat extract; ORAC = oxygen radical absorbance capacity method.**

**Table 3 – Polyphenol content of ChronoCare.**

Composition	% (w/w)
(+)-Catechin	0.1
(-)-Epicatechin	5.5
Procyanidin B1	6.5
Procyanidin C1	1.9
Cinnamtannin A2	0.9

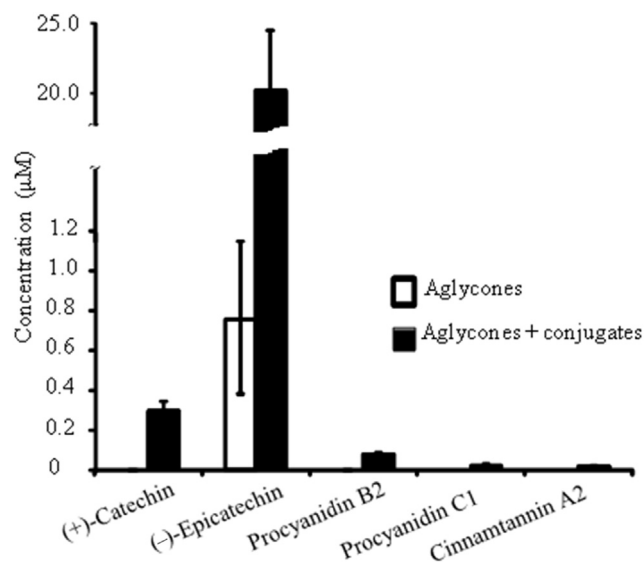
**Table 4 – Recovery of flavan-3-ols from mouse plasma.**

Compound	Recovery (%) <sup>a</sup>
	Mean ± SD
(+)-Catechin	97.3 ± 7.4
(-)-Epicatechin	119.2 ± 17.3
Procyanidin B2	96.9 ± 6.2
Procyanidin C1	71.0 ± 9.2
Cinnamtannin A2	68.7 ± 4.2

<sup>a</sup> Recovery was estimated by spike test.

degradation of procyanidins may also explain the trace detection of procyanidin aglycones. The low doses of individual procyanidin compounds administered compared with the previous study may also explain the trace detection.

HPLC method applied for the determination of procyanidins in biological samples by using fluorescence detection was first reported by Tanaka et al [25]. In that study, only procyanidin B2 and procyanidin C1 isolated from *Cinnamomomi cortex* were evaluated and detected in rat plasma. The fluorescence detection was also reported by Rios et al [26]. In this study, a cocoa beverage made of cocoa powder, which is known for being rich in procyanidins, was given to human participants,



**Figure 6 – Concentrations of procyanidins in mice plasma 1 hour after oral administration of BE at a dose of 250 mg/kg body weight. Flavan-3-ol concentration was measured using HPLC-FLD. BE = black soybean seed coat extract; HPLC-FLD = high performance liquid chromatography coupled with a fluorescence detector.**

and the stabilities of (+)-catechin, (–)-epicatechin, procyanidin B2, and procyanidin C1 in the stomach were evaluated. In our study, (+)-catechin, (–)-epicatechin, procyanidin B2, procyanidin C1, and cinnamtannin A2 from BE were evaluated and detected in mouse plasma. This is the first report on applying and realizing fluorescence detection for the determination of tetramer procyanidin in biological samples, and it proved that HPLC coupled with a fluorescence detector can be used for the elucidation of procyanidin bioavailability.

#### 4. Conclusion

We developed a sensitive and specific HPLC method coupled with a fluorescence detector for the analysis of procyanidins. Our validated analytical method will contribute to the determination of procyanidin compounds in food materials and the elucidation of the bioavailability of procyanidins in future studies.

#### Conflicts of interest

All contributing authors declare no conflicts of interest

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